Managing *Halyomorpha halys*:
Effects of cold tolerance, insecticides, and linguistic uncertainty

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Dedication

To my parents, Mike and Lenore Cira.

Abstract

Managing pests effectively and efficiently requires knowledge about their biology. Likewise, clear communication about scientific research pertaining to management is needed for managers to make well-informed decisions. My research pertains to the biology of the brown marmorated stink bug, *Halyomorpha halys* and communication between scientists about insecticidal categorization. *Halyomorpha halys* is an exotic invasive species in North America. Many horticultural and agricultural plants in North America are at risk for economic damage due to the highly polyphagous nature of this species. Additionally, *H. halys* has become a serious nuisance pest in human-made structures in some areas when, in preparation for winter, they aggregate in sheltered structures. Therefore, factors such as cold and insecticides, which affect *H. halys* distribution and ability to cause crop injury are of particular interest to develop management plans.

I first conducted several studies of cold tolerance on *H. halys* in North America. I found that *H. halys* exhibits a chill-intolerant cold tolerance strategy, that season, sex, and acclimation location affect cold tolerance (i.e., supercooling points), and that laboratory assays of lethal temperature were able to accurately forecast winter mortality in the field. Second, I studied *H. halys* diapause in more depth. I present a protocol for rearing diapausing *H. halys* in the laboratory that exhibit similar cold tolerance (i.e., supercooling points and lower lethal temperatures) as individuals with field-induced diapause. I found that *H. halys* diapause confers greater cold tolerance than a non-diapausing state, and appears to be necessary to survive overwintering. Additionally, I showed diapausing *H. halys* actively feed for at least three weeks post-diapause induction, but feeding is mediated by temperature and adult age. Third, I investigated and critiqued the current paradigm for determining insecticidal efficacy on *H. halys*. I highlighted important sublethal effects of insecticides on feeding that can significantly reduce crop injury without the necessity of high direct mortality to *H. halys.* Lastly, using an interdisciplinary lens I examined linguistic uncertainty as it pertains to categories and descriptors of insecticides. I demonstrated the presence and consequences of

linguistically uncertain terms associated with insecticides and encouraged entomologists to more adequately acknowledge and address these uncertainties in future research.

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Introduction

Pests in agriculture are an inevitable problem (Smith and Kennedy 2002). An agricultural pest is an organism which causes loss (i.e., reduction from maximal attainable yield or quality) of an agricultural product (Walker 1983). Most frequently, pests include weeds, pathogens, and insects. Worldwide, crop destruction due to arthropods was recently estimated to be 18-26% of annual crop production, equating to losses of more than 470 billion USD (Culliney 2014). Management of insect pests takes many forms, and decisions about pest management are made at different scales. Individual growers to federal governments must decide how to manage, what to manage, and when to manage pests. The history of pesticide and insecticide use strongly suggests that integrating management tools is paramount to balancing risks and benefits of pesticides (Metcalf 1980, Casida and Quistad 1998, Kogan 1998, Smith and Kennedy 2002). Pest management decisions are often made based on research about pest and crop biology, and science has an important role to play in improving pest management (Enserink et al. 2013). For example, some studies on pests, crops, and ecosystems contribute to understanding how to effectively and efficiently integrate management options. However, it is incorrect to think science alone can address pest management problems. To ask the why, for who, and at what costs questions about pest management, requires an understanding of how to communicate and weigh risks and benefits of management options and falls outside of strict empirical scientific experimentation.

The components of this dissertation are united as work that contributes to more well-informed pest management decision-making. I investigated two stressors, insecticides and cold, on an exotic invasive species *Halyomorpha halys* (Stål). The profound impact of these two stressors on the biology and management of insects makes them particularly rich grounds for investigation. Insects' lives are fundamentally tied to temperature. No other single environmental factor shapes their developmental rate so greatly (Sharpe and DeMichele 1977). Insecticides are a common management technique not only in agricultural systems, but in urban and home settings as well. In the United States of America in 2012, more than half of the 64 million pounds (34 million pounds)

of insecticides used were applied in agricultural settings, accounting for 3% of the 1,065 pounds applied world-wide in that year (Atwood and Paisley-Jones 2017). My dissertation further contributes to informing decisions on pest management with a chapter examining the nature and consequences of linguistic uncertainty as it pertains to scientific writing, with a focus on categories and descriptors of insecticides. Albert Einstein remarked, "Everything depends on the degree to which words and word-combinations correspond to the world of impression," which makes language a "dangerous source of error and deception" (Einstein 2007, p 449).

My dissertation provides foundational knowledge about *H. halys* cold tolerance and overwintering biology with implications for management (Chapter 1 and 2), highlights important sublethal effects on feeding that can significantly reduce crop injury without the necessity of high direct mortality to *H. halys* (Chapter 3), and encourages entomologists to more adequately acknowledge and address the linguistic uncertainty present in categorization and description of insecticides (Chapter 4).

Insecticides

Management of insect pests in agriculture frequently takes the form of applications of insecticides. The increased use of insecticides around the world in the 21st century clearly has had both positive (reviewed in: Cooper and Dobson 2007) and negative consequences (reviewed in: Pimentel and Burgess 2014). The introduction of decision-making frameworks built from sampling pest densities and calculating economic injury levels contributed to more judicious and targeted use of insecticides (Stern et al. 1959, Pedigo et al. 1986). The idea that complete eradication of pests is unnecessary to maximize profit is central to integrated pest management (IPM). Likewise, a greater awareness of the impact sublethal effects of insecticides has on insect management has arisen (Haynes 1988, Stark and Banks 2003, Desneux et al. 2007).

Insect cold tolerance

What a *cold* temperature is to an insect depends on the species. The temperatures below those to which a species is best adapted are considered cold (Sehnal 1991). Insects

have a range of responses to long-term cold temperature exposure including, but not limited to, changes in: the number of larval instars, size (larger and smaller), morphogenesis pathways, coloration (Sehnal 1991), ability to supercool, ability to survive cold temperatures, and water content (Lee 1991).

Diapause is the most extreme alteration of a morphogenesis pathway. Diapause often is not the result of cold temperatures, but arises in anticipation of cold temperatures (Sehnal 1991). Environmental triggers are experienced by the brain, or in some cases the prothorasic gland (e.g., Numata and Hidaka 1984) and hormonal changes ensue (for review of diapause regulation: Denlinger 2002). In contrast, quiescence, which is also a form of dormancy, is an immediate response to adverse conditions and normal morphogenesis resumes as soon as favorable conditions return (Denlinger 1991, Koštál 2006). An enhanced ability to survive cold temperatures can be dependent on diapause or independent from diapause, but most often, insect diapause confers greater cold tolerance (Denlinger 1991).

Supercooling, or the ability of a liquid to remain unfrozen below its melting point (i.e., when warmed from a frozen state, the temperature at which no ice crystals remain), is common among insects (Lee 1989). The supercooling point, or the temperature at which ice crystals nucleate, is used to measure the extent to which an insect can supercool (Lee 1989). It has been established that supercooling points are not always a sufficient metric to assess an organism's survival from exposure to cold (Renault et al. 2002). Thus, where possible we also measured lower lethal temperatures, defined here as the temperatures at which instantaneous exposure causes mortality.

The relationship between supercooling points, or freezing, and survival has been used to categorize insect cold tolerance. Insect cold tolerance can be divided into three categories: freeze-tolerant, freeze-intolerant, and chill-intolerant (Lee 2010). Freeze-tolerant insects are able to survive ice formation in their body, freeze-intolerant insects survive up until the temperature that induces ice crystals to form, and chill-intolerant insects die from cold injury prior to formation of ice crystals in their body (Lee 2010).

Uncertainty

Science and knowledge are limited by our individual and collective abilities to perceive *and* to communicate our perceptions. Thus, to strive for certainty we must understand the nature of uncertainty in our perceptions, but also as it relates to our use of language in scientific communication. Regan et al. (2008) outlined a taxonomy of uncertainties discussed in the context of ecology. In it, uncertainty is divided into two main types: epistemic and linguistic uncertainty. Epistemic uncertainty comes from a lack of knowledge about a system, while linguistic uncertainty comes from the variably ways in which words are communicated and understood (Regan et al. 2008). Uncertainty can hinder effective decision-making (Milner-Gulland and Shea 2017). However, some authors have attempted to address linguistic uncertainty in their respective field (e.g., Crump et al. 1999; Regan et al. 2000; Eilenberg et al. 2001; Carey and Burgman 2008; Horowitz et al. 2009; Ostry et al. 2011; Herrando-Pérez et al. 2014; Fraser et al. 2015; Milner-Gulland and Shea 2017). Ultimately, authors must understand how linguistic uncertainty effects the strength, validity, reproducibility, and long-term stability of their work to know when and where to allow for linguistic uncertainty to remain.

Halyomorpha halys

Halyomorpha halys, commonly known as the brown marmorated stink bug, is native to East Asia (Hoebeke and Carter 2003). The first record of an established *H. halys* population in North America occurred in 1996 in Allentown, PA (Hoebeke and Carter 2003). Since then, *H. halys* has continued to spread in North America and now has been detected in 43 states and 4 Canadian provinces (www.stopbmsb.org/where-is-bmsb/, accessed May 7, 2017). *Halyomorpha halys* is highly polyphagous, and many horticultural and agricultural plants are at risk for economic damage (Rice et al. 2014, Bergmann et al. 2016). For mid-Atlantic apples, damages in 2010 alone exceeded $37 million (U.S. Apple Association News Release 2011). In addition to being an agricultural pest, due to its overwintering behavior, *H. halys* also has the potential to be a serious nuisance pest in human-made structures (Watanabe et al. 1994).

Thorough reviews on *H. halys* have recently been published. For information about *H. halys* biology, ecology, and management from a United States perspective refer to Rice et al. (2014). Lee et al. (2013) summarize the literature on *H. halys* biology, ecology, and management in China, Japan, and the Republic of Korea. A summary of *H. halys* systematics, key to identifying *H. halys* instars, and general biology can be found in Hoebeke and Carter (2003). Kuhar and Kamminga (2017) summarize the literature on chemical control of *H. halys* and describe current chemical management practices. Following, I briefly summarize the literature on *H. halys* pertinent to my dissertation.

Insecticidal management of Halyomorpha halys

Studies in the summaries of insecticide efficacy in Asia (Lee et al. 2013a) and in the United States of America (Kuhar and Kamminga 2017) largely focus on mortality of *H. halys* after insecticide exposure. Studies which have investigated sublethal effects of insecticides measure changes in adult mobility (Lee et al. 2013c, 2014b, Morrison et al. 2017b) and injury of insecticide-treated apples and peaches (Leskey et al. 2014). However, without providing insecticide-free food Leskey et al. (2014) could not determine the causal mechanism (e.g., behavioral, physiological) for reduced feeding. Injury to insecticide-treated crops have been studied (Funayama 2002, 2012, Leskey et al. 2012b, Aigner et al. 2015), but these experimental designs did not allow for differentiating between lethal and sublethal effects. Pyrethroids are the most widely used insecticides to manage *H. halys* due to their availability, low cost, and ability to cause high mortality (Kuhar and Kamminga 2017). Reliance on pyrethroids is problematic however, due to the unintended detrimental effects the insecticides have on natural enemies (Kuhar and Kamminga 2017).

Cold tolerance of Halyomorpha halys

The literature on *H.* halys cold tolerance is sparse. Results about the triggers of diapause are inconsistent (Watanabe et al. 1978, Watanabe 1979, Yanagi and Hagihara 1980, Niva and Takeda 2002, 2003) and only weak evidence exists to determine the life stage sensitive to photoperiod diapause induction cues (Watanabe 1979). Supercooling points were found to differ between sexes, seasons, and acclimatization locations (Cira et al. 2016), and *H. halys* is considered chill-intolerant (Cira et al. 2016). Other traits of

overwintering *H. halys* such as flight capacity (Wiman et al. 2014b, Lee and Leskey 2015), overwintering site selection (Watanabe et al. 1994, Cambridge et al. 2014, Lee et al. 2014a, Funayama 2015a), nutritional status (Funayama 2015b), response to light (Toyama et al. 2006, 2011) and pheromones (Morrison et al. 2017a), and short-range mechanisms for aggregation (Toyama et al. 2006) have also been investigated.

Chapters

Chapter 1 and 2 pertain to cold tolerance and overwintering of *H. halys.* In these chapters I report the first measurements of *H. halys* cold tolerance in North America. In Chapter 2, I analyzed factors that affect supercooling points and determined how freezing relates to mortality of *H. halys.* I compared populations from Minnesota and Virginia to find supercooling points differed based on season, sex, and acclimation location. Using Minnesota field acclimatized adult *H. halys* I found the species is chill-intolerant. Building off of this, Chapter 2 incudes work related to *H. halys* diapause. I provide a protocol to induce *H. halys* diapause in the laboratory, which results in individuals which exhibit similar cold tolerance (i.e., supercooling points and lower lethal temperatures) as those whose diapause was field-induced. I show that *H. halys* diapause confers greater cold tolerance than a non-diapausing state, and appears to be necessary to survive overwintering. Finally, by measuring salivary flanges of adults post diapause induction I report that diapausing individuals continue to actively feed for at least the first three weeks as an adult, and feeding is mediated by temperature and adult age.

Results from my cold tolerance and diapause studies will contribute to more well-informed pest management by helping to create better parameterized phenology models (e.g., Nielsen et al. 2016), and range models (e.g., Zhu et al. 2012, 2016; Haye et al. 2015; Kriticos et al. 2017), and help to predict spring population levels, behavior, and feeding damage in autumn. The protocol for maintaining a diapausing laboratory colony also provides a foundation from which to explore *H. halys* diapause further. The implications of insect cold tolerance on pest management have been summarized by Bale (2010). Diapause is seen as a promising field for investigating potential novel management techniques (Denlinger 2008). Specifically for *H. halys*, some work has

already begun testing the potential use of pheromones to trap and kill overwintering *H. halys* in structures (Morrison et al. 2017a).

Chapter 3 critiques the dominant paradigm in place to assess efficacy of insecticides on *H. halys*. Predominantly, mortality has come to equate to efficacy. I conducted laboratory assays to examine the effects of insecticides on *H. halys* mortality, molting, and feeding. Increased use of insecticides that are harmful to natural enemies, to manage *H. halys* has led to secondary pest outbreaks and disruptions to IPM programs (Leskey et al. 2012a, Lee et al. 2014b). Thus, insecticides considered less risky to natural enemies were compared to a pyrethroid insecticide and an untreated control.

Quick and complete mortality was found for the pyrethroid, and statistically only sulfoxaflor at 14 days after treatment (DAT) matched this level of mortality. Sulfoxaflor was the only insecticide to cause significant sublethal effects on feeding compared to the control. However, the most accurate estimate of potential injury/individual after insecticide exposure resulted from a combined metric taking into consideration both lethal and sublethal effects. This metric of feeding sites/individual showed all insecticides, except pyrethrins, resulted in significant reductions in feeding sites/individual compared to the control.

This work underscores the importance of assessing sublethal effects of insecticides for *H. halys*. We found that less disruptive insecticides (i.e., those causing less mortality to natural enemies) were capable of significantly reducing feeding flanges from the control. This has strong implications for pest management decision-making, as it offers decision-makers an alternative to insecticides whose use harms natural enemies and results in secondary pest outbreaks.

Entomology is an inherently interdisciplinary field and Chapter 4 reflects an interdisciplinary collaboration through my Science, Technology, and Environmental Policy minor. This chapter brings together ideas about uncertainty, communication, language, and insecticidal categorization to analyze linguistic uncertainty in entomological papers. Scientists deliberately chose and thoroughly research the language they use to propose new concepts or terminology. Often, however, terms may slip into the lexicon through less systematic means. Words may seem to have meanings so

obvious that a mutual understanding of the term may be taken for granted. Yet, individual contexts are diverse across time and space and to assume that a word's meaning is apodictic, or unchanging, even at one point in time, completely overlooks the uncertainties of language. Thus, communication using even just the most common entomological lexicon can fail.

I demonstrate and discuss the presence and consequences of linguistic uncertainty in entomological pest management literature by surveying a subset of entomological peer-reviewed papers, and common entomological reference material. In them, I determined: 1) what explicit definitions, if any, were being employed for the term broad-spectrum insecticide, 2) where no explicit definition was given, what terms were associated and disassociated with broad-spectrum (i.e., inferred definition), 3) which insecticides were considered to be or not be members in the broad-spectrum category, and 4) how the term broad-spectrum was being used according to a qualitative evaluative framework I developed.

I report that broad-spectrum as an insecticide category is vague, ambiguous, relative, and subjective and explain the reason for and consequences of these specific types of uncertainty. I reiterate the need for an increased awareness of linguistic uncertainty in entomological writing, and encourage mindful consideration of where and why linguistically uncertain words are being utilized. I provide suggestions for how to address linguistic uncertainty broadly and also specifically with regard to insecticidal classification. My analysis and exploration of the uncertainty introduced into our science from language can help entomologists communicate more precisely, accurately, and efficiently. Resolving linguistic uncertainties about insecticidal categorization will lead to better science and more well-informed policy and decision-making in pest management contexts.

In summary, my dissertation contributes to an increased understanding of *H. halys* biology as it relates to insecticide- and cold-stress, and reveals areas of uncertainty currently unaddressed in literature about insecticides. My work provides scientific knowledge which allows for more well-informed *H. halys* management decisions to be

made and a qualitative look at the tension linguistic uncertainty creates between quality of information and conciseness of writing when categorizing insecticides.

Chapter 1: Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales

Summary

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is native to eastern Asia and is presently invading North America. Little is known about the exposure to and effects of winter temperatures in newly invaded regions on *H. halys*. The overwintering habitats that this species utilizes vary greatly in their thermal buffering capacity. They naturally overwinter in aggregations beneath loose bark on trees and in cliff-outcroppings, but will also commonly aggregate in buildings. Effects of cold temperatures such as mortality and freezing have yet to be quantified in the invading population. We report that *H. halys* is chill intolerant (i.e., dies before reaching its freezing point), and that the degree of cold tolerance of populations in North America differs by season, sex, and acclimation location. The mean winter supercooling point (± SEM) of individuals acclimated in Minnesota was -17.06°C ± 0.13 and in Virginia was -13.90°C ± 0.09. By using laboratory assays of lower lethal temperatures and ambient air temperature records we accurately forecasted mortality for field experiments in Minnesota and Virginia. Temperature refugia provided by human-built structures are likely crucial for overwintering survival during atypically cold winters and possibly contribute to the northern geographic range expansion of this economically damaging insect in the temperate climates of North America.

Introduction

Halyomorpha halys (Stål), the brown marmorated stink bug, is native to East Asia and has become a severe invasive agricultural pest in the United States (US) on over 33 crop host plants (Bergmann et al. 2015). Damage to Mid-Atlantic apples alone exceeded $37 million in 2010 (U.S. Apple Association News Release 2011). This invading insect has been found in 42 US states since it was first detected in the US in the mid-1990's (Northeastern IPM Center 2015) and is predicted to establish across the entire contiguous United States based on the geographic distribution of hosts and degree day models (Holtz and Kamminga 2010) and ecological niche models (Zhu et al. 2012). These inductive

approaches to modeling however, do not predict fluctuations in population levels, nor do they define the impacts of environmental stresses on populations for ecological and economic risk assessment (Venette et al. 2010). Insects are poikilotherms; ambient temperature strongly affects their development and mortality. Cold often dictates the poleward limits of a species' distribution. To clarify the relationship between cold-stress and mortality, we studied the overwintering strategies and lower lethal temperatures of this invasive species.

The capacity of *H. halys* to survive cold exposure (i.e., cold tolerance) is dependent on the temperatures individuals are *exposed to* and the *effects* of those cold temperatures on an insects' physiology. An observational study in Japan found that winter mortality was reduced by 13.5% for every 1°C rise in mean January and February temperature above 4°C (Kiritani and Biology 2007). Our work expands on these observations by experimentally testing the effects of cold and assessing mortality and cold exposure of *H. halys* that are invading North America. As winter approaches, *H. halys* enters a facultative reproductive diapause (Watanabe et al. 1978) and begins to aggregate in protected areas (Watanabe et al. 1994). In natural settings this species seeks shelter beneath loose bark on trees, but aggregations can also occur in human-made structures (Lee et al. 2014a), which may offer greater thermal protection. We do not know the extent to which the North American population utilizes these overwintering habitats.

In order to examine the effects of cold temperatures on *H. halys* we first determined *H. halys'* cold tolerance strategy. Three broad insect cold tolerance categories exist to describe the relationship between freezing and mortality. Freeze tolerant insects are able to live after the formation of ice within their bodies, freeze intolerant insects live up until the point at which they freeze, and chill intolerant insects die before freezing occurs (Lee 2010). Most temperate insects in the Northern Hemisphere are freeze intolerant (Bale 1991). To avoid freezing, various mechanisms are used, such as the production of cryoprotectants to depress the insect's supercooling point (i.e., the temperature at which body fluids begin to freeze). Overlaying supercooling points and

mortality elucidates the relationship between freezing and mortality and thus an insects' cold tolerance strategy (Hanson et al. 2013).

To further examine the effects of cold temperatures on *H. halys* we compared supercooling points across season, sex, acclimation location, and geographic origin. Acclimation, on both short (Lee et al. 1987, Lee and Denlinger 2010) and long (Salt 1961) time scales, can affect the cold tolerance of an insect. Short-term acclimation, such as rapid cold hardening, can quickly confer an increased cold tolerance (Lee et al. 1987) while long-term acclimation (i.e., acclimatization), such as from changing environmental temperature and photoperiod cues, leads to seasonal differences in cold tolerance (Salt 1961). The climate to which an individual is acclimated can also alter the degree of cold tolerance an insect can achieve (Bradshaw 2010). Extreme weather events have the potential to cause high mortality when insects are inadequately acclimated. During the winter of 2013–2014 much of North America experienced what was commonly referred to as the "polar vortex," i.e., low pressure and cold arctic air which escaped the typical circulation pattern that generally revolves around a low pressure system over the North Pole. The uncommonly low temperatures experienced during the polar vortex provided a rare opportunity to measure field mortality of *H. halys* under extreme conditions.

Field-acclimated *H. halys* from Minnesota were used to determine the species' cold tolerance strategy and field-acclimated *H. halys* from Minnesota, Virginia, and West Virginia were used to investigate factors affecting supercooling points. As *H. halys* occurs in temperate climates we hypothesized that *H. halys* would be freeze intolerant, that the temperatures at which *H. halys* freezes would vary according to season, sex, and acclimation location, but not geographic origin. We also assessed the degree to which our laboratory measurements could deductively forecast the winter field mortality in Minnesota and Virginia in two common types of overwintering habitats.

Materials and methods

Insects

Virginia-sourced & Minnesota-acclimated insects

Halyomorpha halys eggs were shipped overnight from Blacksburg, VA to St. Paul, MN on July 2, 2013. Eggs were maintained at 25°C 16L:8D in a growth chamber until the insects molted into second instars. Second instar nymphs were placed in mesh cages 38cm x 38cm x 61cm (BioQuip, Rancho Dominguez, CA) within a larger wire screen enclosure and provisioned with potted snap bean plants (*Phaseolus vulgaris* L. cv 'Romano Bush #14'), dried raw organic sunflower (*Helianthus annus* L.) seeds and soybean (*Glycine max* (L.) Merr.) seeds as needed. These cages were maintained outdoors on the St. Paul campus of the University of Minnesota (44°59'20.7"N 93°11'10.6"W, elev. 300m). On October 18, 2013 insects were transferred into circular plastic dishes (18.5cm diameter x 8cm high) (Pioneer Plastics, Inc., North Dixon, KY) with a 25 x 89cm piece of cotton canvas provisioned with dry organic soybean seeds and placed into an unheated shed on the St. Paul campus of the University of Minnesota to mimic where and how the insects might otherwise aggregate.

Minnesota-sourced & Minnesota-acclimated insects

Eggs, nymphs, and adults were maintained as above; however, eggs originated from a laboratory colony at the University of Minnesota which had been maintained since 2012. Additionally, adults were transferred from outdoors to either an unheated shed or a walk-in cooler on October 30, 2014. Mean walk-in cooler temperature ± standard error of the mean was 4.52°C ± 0.001 with constant darkness as a constant temperature control.

Virginia-sourced & Virginia-acclimated insects

From June to October in 2012 and 2013, adult *H. halys* were collected from sweet corn and unmanaged trees located on a private farm, Garrett Farms in Glenvar, VA. In October of each year, a sample of >2000 adults was collected and placed in artificial overwintering habitats that consisted of eight plastic 18.9 liter buckets (Encore Plastics Corp., Byesville, OH) packed tightly with 12.7 mm thick foam pipe insulation (Thermwell Products, Inc. Mahwah, NJ). A 10 cm diameter ventilation hole was made into the side of each bucket, and covered with screen to prevent insect escape. Buckets

were maintained outdoors in Blacksburg, VA (37° 12.417' N, 80°35.513' W, 616 m elev.) throughout the winter. This approach allowed for access to a population of insects that were exposed to ambient winter temperatures.

West Virginia-sourced & Virginia-acclimated insects

On November 1, 2012 overwintering adult *H. halys* were collected from a private facility in the panhandle of West Virginia and shipped overnight to Blacksburg, VA. They were maintained in the same artificial overwintering habitat as described above.

Cold tolerance strategy and supercooling point testing methods

Minnesota

Supercooling points and lower lethal temperatures were measured by using contact thermocouple thermometry where individual adults were placed in close proximity to coiled copper-constantan thermocouples (e.g., Hanson and Venette 2013) that were attached to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Temperatures were recorded once per second and logged by using Tracer-DAQ software (Measurement Computing, Norton, MA). We identified the lowest temperature reached before the exotherm, or spontaneous release of heat indicative of a phase change from liquid to solid, to denote an individual's supercooling point.

In 2013 the insect and thermocouple were confined in a 20 or 35ml syringe (Monoject syringes with leur lock tip) that was placed at the center of a 20 x 20 x 20cm polystyrene cube and then into a -80°C freezer where the insects cooled at a realized rate of -0.82ºC (± 0.008°C) per minute according to Carrillo et al. (2004). Supercooling points from 14 males and 14 females in fall (October 9-10, 2013), and 10 males and 9 females in winter (December 7-9, 2013) were measured. Additionally on December 7-9, 2013, for the determination of cold tolerance strategy, 85 Virginia-sourced, Minnesota-acclimated adult *H. halys* were randomly assigned to one of five temperature treatments (-20, -15, -10, -5°C, or 25°C), so n=17 adults per temperature. Insects were cooled until they reached the desired temperature as tracked though the above thermocouple method, removed immediately, and allowed to warm to room temperature. After warming, insects were transferred to individual plastic cups provisioned with water and dry organic soybean seeds and monitored daily for mortality for four days. One day after treatment,

all insects which were going to recover from chilling had done so and mortality measurements from one day after treatment were used for statistical analysis. Mortality of the insect was defined as a lack of any movement after being gently prodded with a small paintbrush; moribund insects, defined as having the inability to right itself after ~10 s or the inability to walk, were considered dead for the purposes of analysis.

In 2014 the insect and thermocouple were confined in an 18x150mm (ODxL) Kimax glass test tube, stabilized with one sheet (11.18 x 21.34cm) of Kimtech delicate task wipers, and a rubber test tube stopper with a 5mm hole. This apparatus was placed in a refrigerated bath of silicon 180 oil (Thermo Fischer Scientific A40, Waltham MA) at room temperature and chilled at a rate of -0.95ºC (± 0.003°C) per minute. Eighty five Minnesota-sourced, Minnesota-acclimated insects were chilled according to the above methods for cold tolerance strategy on December 10, 2014.

Virginia

Starting in June of 2012 samples of 10 male and 10 female *H. halys* adults were tested at approximately two month intervals throughout the year to determine supercooling point. Supercooling points were determined by placing adult insects on an apparatus where a copper-constantan thermocouple (Omega Technologies, Stamford, CT) was placed on the ventral and dorsal side of each insect. Weighted aluminum blocks were used to apply pressure and ensure contact between the insect and the thermocouple. Additionally, a small amount (<0.1 mL) of thermal grease (zinc oxide) was placed on the tip of the thermocouple to assist with any gaps in contact. DaqView (MC Measurement Computing, Norton, MA) measured temperatures generated from a 50/50 water and ethanol mixture in a refrigerated water bath (Fisher Scientific Isotemp, Waltham, MA) in circulation with a cold plate (Stir-Kool Cold Plate [SK-31], Thermoelectrics Unlimited, Wimington, DE). This setup was able to cool the plate down to -28°C. The arena with insects was placed on the cold plate with a series of foam and aluminum blocks to reduce the temperature around 0.3°C per minute until the exotherm occurred (Bentz and Mullins 1999).

Field temperature observations

In Minnesota, air temperatures were recorded at 15 minute intervals from October 18, 2013 to March 5, 2014 with an Hobo U12 4-External channel outdoor/industrial data logger (Onset Computing, Bourne, MA) and October 30, 2014 to February 26, 2015 via an U12 Temp/RH/2 External Channel Logger (Onset Computing, Bourne, MA).Temperature probes or loggers were placed next to insect cages in an unheated shed in Minnesota.

In Virginia, minimum daily air temperatures were collected from a NOAA weather station that was 3.75 km from where the insects were stored.

Statistics

Cold tolerance strategy

All statistics were run using R version 3.2.0 (R Core Team 2014) in RStudio version 0.98.1102 (RStudio Team 2016) and for all analyses an α value of 0.05 was used. Modified "survival curves," where temperature substituted for time, were created to describe the probability that *H. halys* acclimated in Minnesota and tested in winter would freeze or die at a particular temperature. Our analyses formally considered censoring (i.e., incomplete information about supercooling points or lethal temperatures) of individuals in the study. Insects that died were interval censored because death occurred between room temperature and the temperature at which the insects were removed. Insects that remained alive were considered right censored (i.e., they could survive the coldest temperature to which they were exposed but would likely die if exposed to a colder temperature). Insects which did not freeze were right censored as they would be expected to freeze at a temperature colder than when they were removed from chilling. The following R packages were used to estimate survival functions with censored data: *survival* (Therneau 2015) to create a survival object, and *interval* (Fay and Shaw 2010) to calculate the non-parametric maximum likelihood estimate for the distribution from interval censored data. Curves were fitted to the binomial data for mortality and cumulative supercooling points. A Kaplan-Meier-Turnbull non-parametric model was used for both years. Curves for freezing and mortality were compared within years via the *icfit* command from the interval package.

Supercooling point comparisons

Some of our supercooling points seemed unusually warm, which could occur if a water-bearing substance (e.g., feces) triggered exogenous ice formation as the insect was cooled. We removed any supercooling point which was more than two standard deviations warmer than the overall mean observed for the entire data set. Seven observations ranging from -3.27 to -2.16 °C were removed, and the remaining data (n =188 adults) were used for subsequent analyses.

Supercooling points for *H. halys* that were collected from West Virginia and Virginia and acclimated in Virginia were compared. A Shapiro-Wilk test for normality of residuals (W = 0.99; df = 11, 86; $P = 0.90$) and a Levene test for homogeneity of variance across groups ($F = 0.99$; df = 11, 86; $P = 0.46$) confirmed no violations of ANOVA assumptions so a fully crossed three-factor analysis of variance was performed on untransformed data with location, season, and sex as main effects. This ANOVA revealed no significant main effects of location ($F = 2.40$; df = 1, 86; $P = 0.13$) nor interaction effects between season and location ($F = 2.32$; df = 2, 86; $P = 0.10$), sex and location ($F = 1.61$; df = 1, 86; $P = 0.21$), or the three-way interaction between season, sex, and location ($F = 0.41$; df = 2, 86; $P = 0.66$); therefore, data for individuals originally from West Virginia and Virginia and acclimated in Virginia were combined for analysis of season, sex, and acclimation location.

In in all analyses, season was defined by the month the insect was tested and followed standard climatological definitions: Spring = April or May; summer = June, July, or August; fall = September, October, November; winter = December or February. Acclimation location was either Blacksburg, VA (37° 12.417’ N, 80°35.513’ W, 616 m elev.) or St. Paul MN (44° 59' 18.9672" N, -93° 10' 51.06" W, 299 m elev.). Where geographic origin was considered, location was determined by where eggs were laid in the field, either Blacksburg, VA (37° 12.417’ N, 80°35.513’ W, 616 m elev.) or Harper's Ferry, WV (39° 19.31’ N, 77° 44.37 W, 489 m elev.). Sex was determined by visual inspection of the genitalia on the posterior ventral surface of the insect’s abdomen.

Season, sex, and acclimation location effect on supercooling points

To test the hypotheses that season, sex, and acclimation location affect cold hardiness we ran a fully crossed three-factor ANOVA (season × sex × location) of supercooling points from Minnesota and Virginia adults from fall and winter. The data met assumptions of normality (Shapiro-Wilk W = 0.98; df = 9, 111; $P = 0.12$) but not homoscedasticity (Levene's test: $F = 2.03$; df = 9, 111; $P = 0.04$). A Box-Cox transformation ($y_\lambda = (y^\lambda - 1)/ \lambda$) where $\lambda = 2.0$ was used and all analyses were done on transformed data. Sex and all interactions were not significant and were pooled for this analysis. Despite the interaction of season and acclimation location being non-significant we were still interested in comparing mean supercooling points between states and seasons to test the hypothesis that season matters to acclimation, and acclimation location matters to mean supercooling points. Tukey's HSD was used to determine the significance of each pairing of interest.

Geographic origin effect on supercooling points

A third hypothesis, that geographic origin matters to cold hardiness, was tested with a fully crossed three-factor analysis of variance (origin × season × sex) for adults that came from eggs laid either in West Virginia or Virginia but were all acclimated as adults in Virginia then tested in fall, winter, and spring. The data satisfied assumptions of homoscedasticity (Levene's test ($F = 1.35$; df = 11, 81; $P = 0.21$) and normality (Shapiro-Wilk (W = 0. 99; df = 11, 81; $P = 0.50$). The interaction of season and sex was significant ($F = 3.37$; df = 2, 81; $P = 0.04$) so sexes were not pooled. Tukey's HSD was used to determine the significance of season by sex by acclimation location.

Predicted field mortality

Predicted field mortality in Minnesota was determined through the survival analysis as described above. Where only supercooling points have been measured and not mortality, an estimate of mortality can still be predicted from the cumulative distribution of supercooling points. With a chill intolerant species we can be confident that individuals that had frozen would be dead, even though mortality likely occurred before freezing began. Consequently, mortality estimates that are based on the cumulative relative frequency of supercooling points (i.e., the cumulative proportion of individuals

that gave an exotherm when cooled to a specific temperature) would predict a species to be able to survive colder temperatures than in reality. This risk averse estimate is still useful however, especially in cases when only supercooling points are known, because observed mortality is unlikely to be less than predicted mortality (unless insects are in a microhabitat which is not exposed to the recorded air temperatures). Based on the cumulative relative frequency of supercooling points for adult *H. halys* acclimated as adults in Virginia and tested in winter, we fit a three-factor Weibull curve which can be used to forecast field mortality in Virginia.

Results and discussion

Cold tolerance strategy

Until now, the specific effects of cold temperatures on *H. halys* were unknown. Contrary to our original hypothesis that *H. halys* would be freeze intolerant, able to survive all temperatures up until the point of freezing, we found that *H. halys* is a chill intolerant species; adults died at significantly warmer temperatures than they froze in 2013 ($Z = 2.50$, $P = 0.01$) and 2014 ($Z = 3.99$, $P < 0.001$) (Fig. 1.1). In the course of our lower lethal temperature experiments, we found that no individual survived if it froze (Table 1.1), ruling out freeze tolerance as a strategy. In the groups of insects that were cooled to -5 or -10°C, no individuals froze but a portion of the insects died (Table 1.1), further supporting our assessment that *H. halys* is chill intolerant. Although the temperatures that caused *H. halys* to freeze or die in Minnesota varied between years, the cold tolerance strategy, i.e., chill intolerance, remained the same.

Season, sex, and acclimation location effects on supercooling points

Halyomorpha halys mediates exposure to cold in a variety of ways. Previous research demonstrated that *H. halys* enters diapause, aggregates, and seeks shelter. Our work highlights an additional means by which *H. halys* reduces exposure to lethal temperatures, by acclimating seasonally and thus lowering the temperatures which would result in mortality. Seasonal collections of adults in Minnesota and Virginia showed that supercooling points changed with season (Two-way ANOVA, $F = 63.03$; df = 1, 111; $P < 0.001$). Mean supercooling points (± SEM) were relatively high in even in winter.

Pooling Minnesota and Virginia supercooling points, the means were -9.43°C ± 0.42 in summer, -15.40°C ± 0.43 in fall, and -16.11°C ± 0.37 in winter. Supercooling points of adults acclimated in Minnesota and Virginia were significantly lower in fall and winter than summer (Fig. 1.2) which supports a seasonal acclimation to cold temperatures that is typical of many temperate insects (Bale and Hayward 2010).

Other effects of cold changed based on acclimation location. For example, supercooling points significantly changed according to acclimation location (Two-way ANOVA, $F = 28.74$; df = 1, 111; $P < 0.001$). Mean supercooling point (±SEM) across seasons in Virginia was -10.86°C ± 0.40, and in Minnesota -16.93°C ± 0.23. We found that *H. halys* acclimated in Minnesota became more cold tolerant earlier in the year as compared to those acclimated in Virginia, likely due to photoperiodic and temperature differences between latitudes (Fig. 1.2).

Our simultaneous comparison of the effects of season and acclimation location show that beginning in summer we saw a steady reduction in supercooling points by season in Virginia (Fig. 1.2). In Minnesota we saw no change in supercooling points from fall to winter. In fall, individuals acclimated as nymphs and adults in Virginia (-13.06°C ± 0.14) had warmer mean supercooling points than in Minnesota (-16.85°C ± 0.08). No statistical difference was found in winter between Virginia (-13.90°C ± 0.09) and Minnesota (-17.06°C ± 0.13). Sex did not significantly impact the supercooling point (Two-way ANOVA, $F = 0.11$; df = 1, 111; $P = 0.73$) and no significant two or three way interactions occurred between location, season, and sex (Fig. 1.2).

Geographic origin effect on supercooling points

The supercooling points of adult *H. halys* collected in West Virginia and Virginia and held outside in Virginia were not different (Two-way ANOVA, $F = 1.21$; df = 1, 81; $P = 0.27$). This supports our hypothesis that acclimation location, not geographic origin, has a stronger effect on the cold tolerance of *H. halys.* Supercooling points for individuals from both locations changed seasonally (Two-way ANOVA, $F = 3.87$; df = 2, 81; $P = 0.02$), though this effect was not the same for both sexes (Two-way ANOVA, interaction of sex and season: $F = 3.37$; df = 2, 81; $P = 0.04$). The sex of the insect did

not have a consistent effect on supercooling point (Two-way ANOVA, $F = 3.83$; df = 1, 81; $P = 0.05$) (Fig. 1.3).

Females from the eastern United States exhibited less seasonal change than males, which suggests that once acclimated in fall, females do not continue to acclimate, but males do. West Virginia males in the fall had the warmest mean supercooling points (-7.93°C ± 1.93) and Virginia males in winter had the coldest (-15.72°C ± 0.56). Mean supercooling points for all other season, sex, and geographic location combinations were not significantly different from each other (Fig. 1.3).

No interactions occurred between geographic origin and season ($F = 1.12$; df = 2, 81; $P = 0.33$), origin and sex ($F = 1.84$; df = 1, 81; $P = 0.16$), or between origin, season, and sex ($F = 0.37$; df = 2, 81; $P = 0.69$). In each season, the mean SCPs between West Virginia and Virginia were statistically equivalent (Fig. 1.3).

Predicted and observed field mortality of H. halys

Supercooling points are relatively easy to measure and give clear results, so they are often a starting point for cold hardiness experiements when specimens are limited (Morey et al. 2012). The cumulative frequency distribution of supercooling points can also provide estimates of mortality for a chill-intolerant or freeze-intolerant species after accute exposure to a specified temperature. It should be noted, however, that these forecasts do not apply to chronic exposures to cold nor do they account for other cuases of winter mortality such as starvation or desiccation. For chill-intolerant species, we know that the estimate will be biased, consistently forecasting less mortality (i.e., greater survivorship) than will be oberved (Fig. 1.1). Despite this, forecasts from a cumulative supercooling point curve can still prove to be useful when making conservative estimates of winter mortality (Table 1.4).

Using field temperature data, laboratory determined cumulative frequency distribution of supercooling points from *H. halys* in Virginia (Fig. 1.4), and laboratory mortality measurements from *H. halys* in Minnesota (Fig. 1.1), we accurately predicted field mortality in Minnesota and Virginia before and after low temperature events (Table 1.2). In December 2014, the expected mortality for overwintering *H. halys* in an unheated shed in Minnesota was greater numerically than our observed mortality;

however, observed mortality at this time falls within the 95% confidence interval for expected mortality (0 to 44%). Our laboratory measurements provided an accurate way to forecast winter field mortality of unheated *H. halys* aggregations in Minnesota and Virginia; however, *H. halys* utilizes an array of overwintering habitats. The tendency of *H. halys* to aggregate in thermally-protected areas, such as human-made structures, means the unheated microclimates into which our experimental insects were placed are not representative of the whole population. Following the extreme low temperatures of the polar vortex of 2014, *H. halys* were still present in the summer of 2014 (Herbert 2014). This suggests that while lethal temperatures were reached across much of the United States, exposure to those temperatures did not occur across the population. Refugia from cold temperatures for *H. halys* are likely contributing to the northern geographic range expansion and ability to survive winter temperatures in North America. Further data should be gathered on a range of overwintering microclimate environments to determine the exposure of *H. halys* to cold temperatures and to better forecast overall overwintering mortality.

In 2014 a subset of insects were maintained in a walk-in cooler as a control group. The temperature in the walk-in cooler was below *H.halys* lower developmental threshold (14.17°C) (Nielsen et al. 2008a) and never reached a point at which we expected mortality; however, in December and January we observed 5% and 15% mortality, which was greater than the 0% (0 – 4%; 95% CI) that was expected when the coldest temperatures these inects experienced was 4.3°C (Table 1.2). This indicates that other factors, possibly dessication or starvation, are contributing to overwintering mortality in addition to cold temperatures. Future directions could include questions about multiple stressors and their effects on overwintering mortality. Additional factors such as nutrition (Gash and Bale 1985), rate of cooling (Baust and Rojas 1985), life stage (Lee 1991), and temperature fluctuations (Salt 1961) can also contribute to cold tolerance and could be investigated in future studies.

Diapause can enhance insect cold tolerance but does not always necissarily do so (Denlinger 1991). *Halyomorpha halys* is known to go into a reproductive diapause before overwintering (Niva and Takeda 2003, Nielsen and Hamilton 2009b). However, nothing

is known about the relationship between diapause and cold hardiness in *H. halys*. A limitation of our studies was that no dissections were done to positively determine if individuals were in diapause when being tested. Nevertheless, because *H. halys* is predicted to be univoltine in Mid-Atlantic states we believe that, by the time winter testing occurred, insects would have received the cues needed to enter diapause (Nielsen and Hamilton 2009b). More work is needed to understand how and when *H. halys* enters diapause and what effects, if any, diapause has on *H. halys* overwintering capabilities. Our work provides new insight into the exposure and effects of cold temperatures on *H. halys.* Studying cold stress on this species has the potential to illuminate new modes of management. *Halyomorpha halys* has become a severe pest in parts of North America. This exotic invasive species causes severe economic damage to many crops and seeks shelter, sometimes in homes, to overwinter, making it an agricultural pest a well as a structural and nuisance pest. A lack of exposure to lethal temperatures when *H. halys* overwinters in thermally buffered areas not only affects the potential geographic range of *H. halys*, it enlarges management problems and solutions beyond typical agricultural settings. More studies are needed to: Quantify the proportion of *H. halys* in specific types of overwintering sites, model the relationship between those microhabitats and the reported air temperature, and investigate other stressors leading up to and through winter that may contribute to mortality.

Tables and Figures

Table 1.1. Proportion mortality ± SEM of adult *H. halys* acclimated outdoors in Minnesota in 2013 and 2014 and exposed to one of five temperatures. Numbers in parentheses indicate the total number of adults that were either chilled (unfrozen) or frozen upon reaching the target temperature.

	2013		2014	
Temperature (°C)	Chilled	Frozen	Chilled	Frozen
-20	-- (0)	1.00 ± 0.00 (17)	1.00 ± 0.00 (1)	1.00 ± 0.00 (16)
-15	0.64 ± 0.12 (14)	1.00 ± 0.00 (3)	0.13 ± 0.08 (16)	1.00 ± 0.00 (1)
-10	0.18 ± 0.09 (17)	-- (0)	0.29 ± 0.11 (17)	-- (0)
-5	0.18 ± 0.09 (17)	-- (0)	0.24 ± 0.10 (17)	-- (0)
25 (control)	0.00 ± 0.00 (17)	-- (0)	0.00 ± 0.00 (17)	-- (0)

Table 1.2. Observed and expected *H. halys* winter mortality. Minimum temperatures reached in Minnesota and Virginia in the winter of 2013-2014 and 2014-2015, expected mortality of *H. halys* adults for recorded temperatures, observed mortality.

		2013		2014	
		MN unheated shed	VA unheated 19 liter bucket	MN unheated shed	MN walk-in cooler
Dec.	Min. temp. reached (°C)	5.5	15.0	-12.09	4.30
	Expected mort. (95% CI)[a]	0.0% (0-4%)	0.0%	27.7% (0-44%)	0.0% (0-4%)
	Observed mort.[b]	0.01 %	N/A	0.0%	5.0%
	Sample size	86	N/A	85	20
Jan.	Min. temp. reached (°C)	-22.6	-20.7	-17.86	4.35
	Expected mort. (95% CI)[a]	99% (53-100%)	99%	99% (17-100%)	0.0% (0-4%)
	Observed mort.[c]	100%	97.6%	100%	15.0%
	Sample size	92	2654[d]	20	20

[a] Based on the minimum temperature reached before testing dates from each location and the mortality curves from Fig. 1.1 for Minnesota (MN) and Fig. 1.4 for Virginia (VA).
[b] Mortality determined on December 6, 2013 and December 10, 2014 in Minnesota.
[c] Mortality determined on January 6, 2014 and January 10, 2015 in Minnesota and February 12, 2014 in Virginia.
[d] This large sample size was possible in Virginia where *H. halys* is abundant.

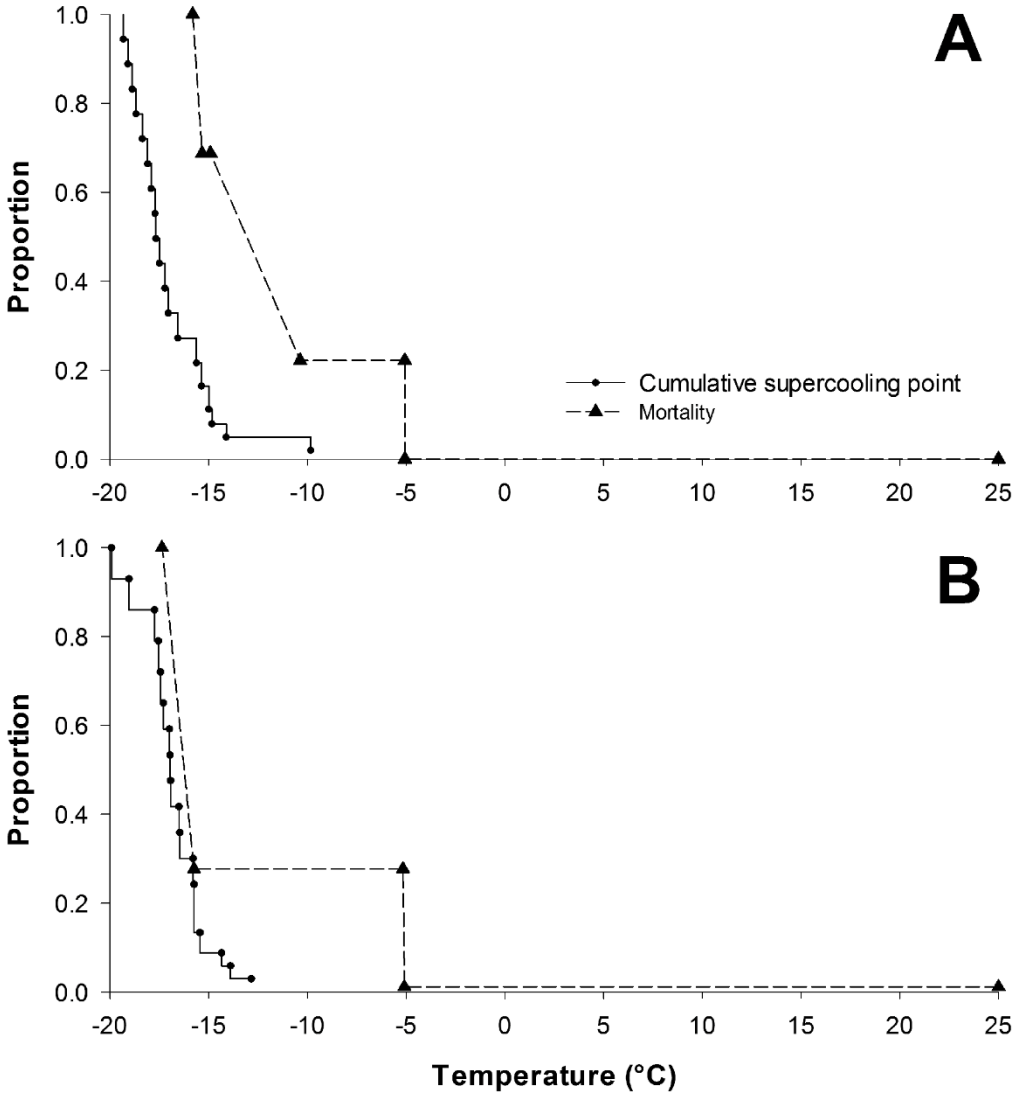

Figure 1.1. Observed cumulative supercooling point distribution and mortality distribution for field-acclimated adult *Halyomorpha halys* in Minnesota in **A)** December 2013 **B)** December 2014. Extrapolation between observed data calculated with right and interval censored survival analysis.

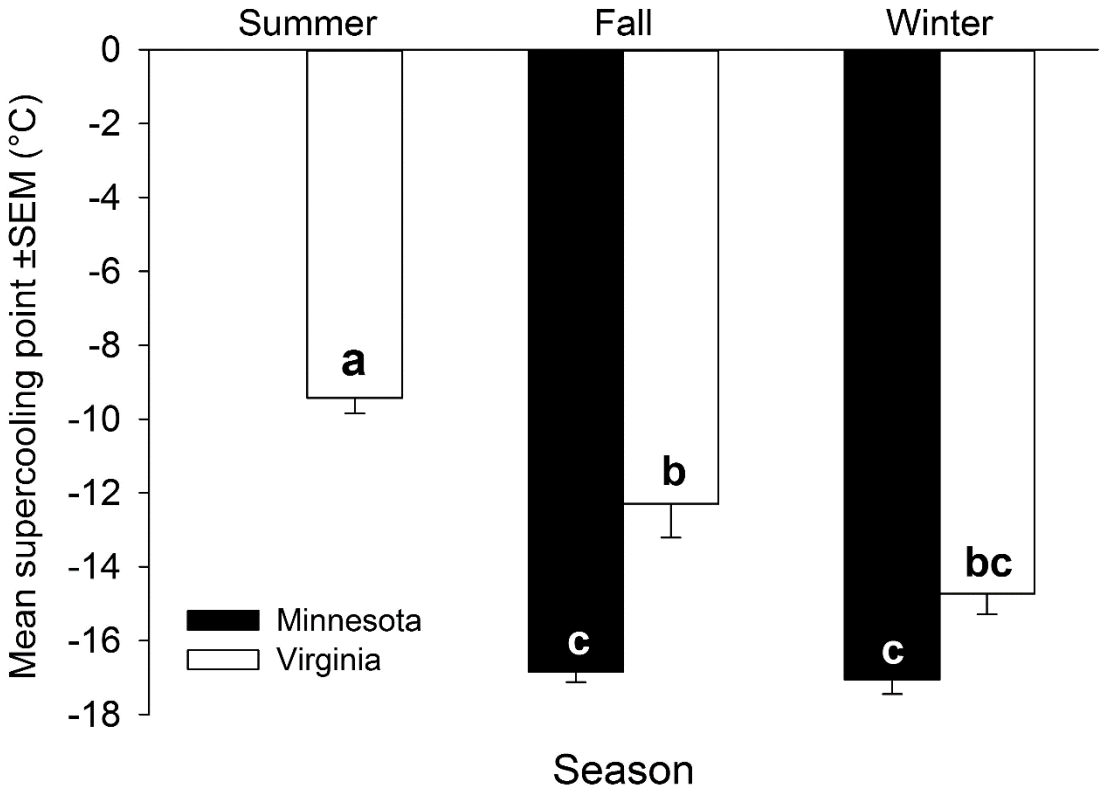

Figure 1.2. Mean supercooling points of adult *H. halys* field-acclimated either in Virginia or Minnesota. Individuals remained outdoors, experiencing temperature and photoperiodic cues, until the time of testing. Error bars indicate standard error of the mean. Statistics were run on transformed data and bars with the same letter are not statistically different ($P > 0.05$; Tukey's HSD).

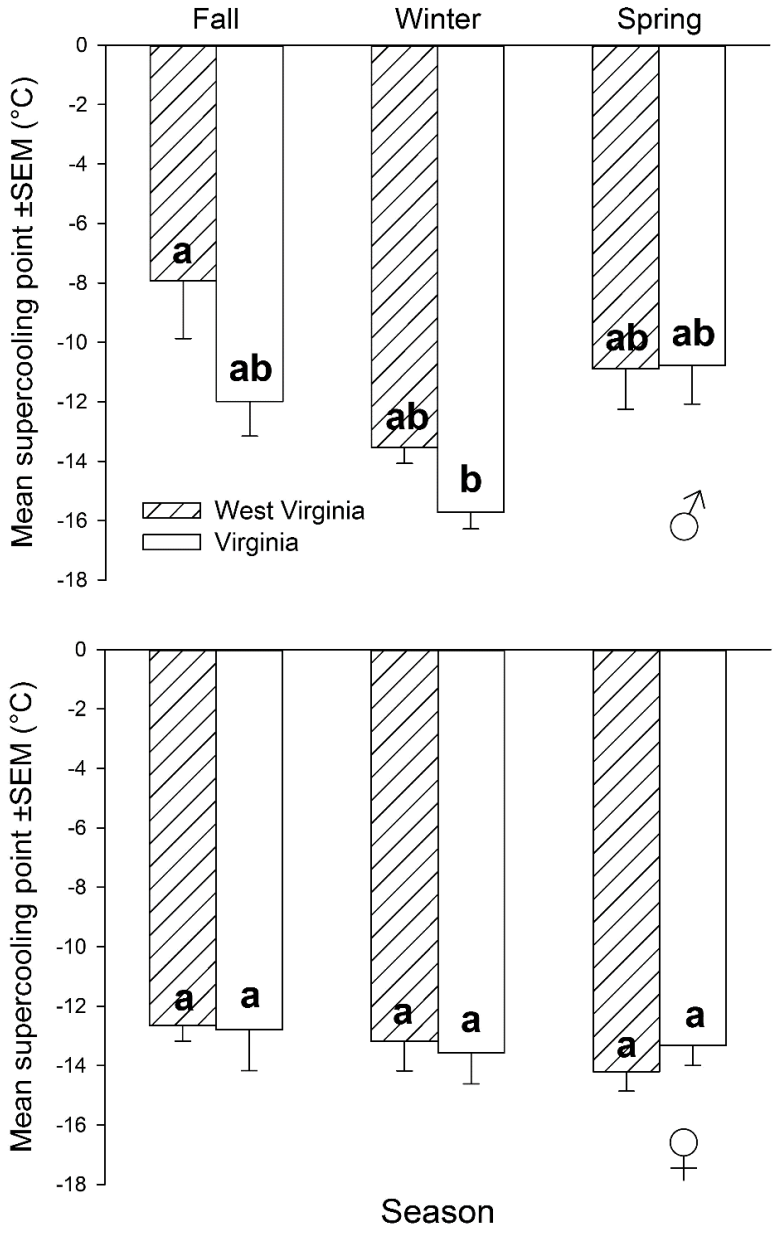

Figure 1.3. Mean supercooling points of *H. halys* adults originating from either West Virginia or Virginia and field-acclimated as adults in Virginia (Top panel) Male, (Bottom panel) Female. Individuals remained outdoors, experiencing temperature and photoperiodic cues, until the time of testing. Error bars indicate standard error of the mean. Across both panels bars with the same letter are not statistically different ($P > 0.05$; Tukey's HSD).

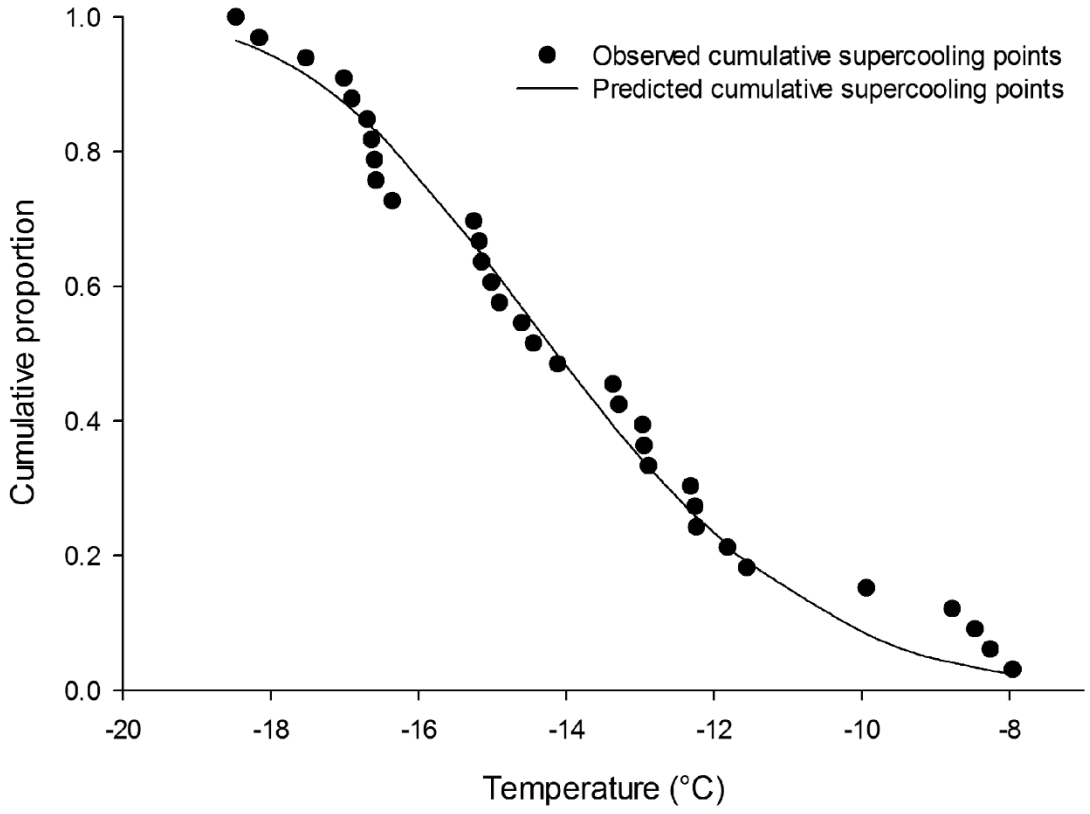

Figure 1.4. Predicted and observed cumulative supercooling point distribution for field-acclimated adult *H. halys* in Virginia in winter. Predicted curve calculated with a Weibull distribution.

Chapter 2: *Halyomorpha halys* diapause induction and effects on cold tolerance and feeding

Summary

We investigated *Halyomorpha halys* (Stål) winter dormancy to confirm a state of diapause and to better understand how this state affects cold tolerance, supercooling points, overwintering mortality, and autumn feeding. Individuals were reared in either laboratory, semi-field, or field conditions. Supercooling points, ovary development, lower lethal temperatures, mass, and feeding were measured. We found that laboratory rearing conditions of 20°C 12L:12D and 20°C 8L:16D induced diapause, as characterized by a lack of ovarian development in all females examined. Laboratory-induction of diapause resulted in statistically similar cold acclimation to field-induction, as measured by supercooling points, but only in laboratory individuals >34 days after adult eclosion, indicative of diapause intensity increasing over time. Increasing diapause intensity was further supported by monthly measurements of supercooling points from October to March. Supercooling points of non-diapausing individuals were significantly higher than laboratory and field diapausing individuals older than 34 days from adult eclosion. Following diapause induction, changes to temperature and photoperiod significantly affected the proportion of individual that fed, the quantity of feeding flanges produced, and the proportion of individuals that reached their supercooling point at a given temperature, but had no significant effect on lower lethal temperatures. This indicates a degree of plasticity of diapausing *H. halys* in some traits but not all. Lower lethal temperatures were not statistically different between field- and laboratory-induced diapausing individuals and occurred at significantly lower temperatures than non-diapausing individuals. Long-term monitoring of diapausing *H. halys* found no ovarian development from October to March. In summary, we provide a protocol for rearing diapausing *H. halys* in the laboratory that exhibit similar cold tolerance (i.e., supercooling points and lower lethal temperatures) as individuals with field-induced diapause. We found that *H. halys* diapause confers greater cold tolerance than a non-diapausing state, and appears to be necessary to survive overwintering. Additionally, we show that

diapausing individuals actively feed for at least three weeks post-diapause induction, but feeding is mediated by temperature and adult age.

Introduction

Halyomorpha halys (Stål) has spread from its native range in East Asia into Europe, and North and South America (Hoebeke and Carter 2003, Wermelinger et al. 2008, Faúndez and Rider 2017). A wide array of host plants, including many of economic importance, are at risk of *H. halys* damage (Hoebeke and Carter 2003, Bergmann et al. 2016). *Halyomorpha halys* can also become a serious nuisance pest when adults seek overwintering sites in human dwellings (Watanabe et al. 1994, Hoebeke and Carter 2003, Inkley 2012). Aggregating in protected structures is one way that *H. halys* copes with thermally unfavorable periods that occur in temperate climates. *Halyomorpha halys* goes through other seasonal changes, such as an enhanced ability to supercool (i.e., lowering the temperature at which body fluids begin to freeze) as winter approaches. Lower supercooling points are beneficial because, as a chill intolerant species, individuals die at temperatures warmer then the temperature at which they freeze (Cira et al. 2016). Thus, if freezing occurs at a lower temperature mortality potentially can also occur at lower temperatures. In winter, *H. halys* is also reported to cease feeding (Nielsen et al. 2016) and enter a facultative reproductive diapause (Watanabe et al. 1978, Nielsen and Hamilton 2009a).

Diapause is a dynamic process that allows an insect to better cope with adverse conditions, such as cold and starvation, and synchronize growth across a population (Denlinger 1991). When diapause is induced, an arrested developmental pathway is triggered. Diapause commonly manifests as lowered metabolism and cessation of feeding and reproduction (Tauber et al. 1986). Koštál (2006) outlines three main phases of diapause: pre-diapause, diapause, and post-diapause, which are described here in further detail. Pre-diapause is further sub-divided into two categories: *induction* is the perception of cues in the sensitive phase that induce a diapausing ontogenetic path, and *preparation* is when diapause has been induced but not yet initiated. During pre-diapause preparation, individuals can continue to develop and progress through physiological and behavioral

preparations for diapause. In the diapause phase, three sub-phases occur: *initiation* is when morphogenesis ceases, but physiological and behavioral preparations for adverse conditions, such as feeding, may still occur; *maintenance* is when individuals remain insensitive to diapause termination cues; and *termination* starts when diapause intensity minimizes to the point that development, or the potential to develop, resumes.

For the family Pentatomidae, most species diapause as adults and induction is principally driven by photoperiod (Saulich and Musolin 2014). Inconsistent results have been reported for laboratory rearing regimes (i.e., temperature and photoperiod) that do and do not induce *H. halys* diapause. Regimes using 25°C and 16L:8D photoperiod have produced both complete non-diapausing populations (Watanabe 1979), and populations with a portion of diapausing individuals (Niva and Takeda 2002, 2003). Similarly, at 25°C and 14L:10D studies report *H. halys* was diapausing (Niva and Takeda 2002), and also not diapausing (Watanabe et al. 1978, Watanabe 1979), and the same temperature with 12L:12D induced diapause in two studies (Watanabe 1979, Niva and Takeda 2003) and partially in another (Watanabe 1979). At 20°C, 16L:8D resulted in a portion of individuals in diapause while 11L:13D resulted in all individuals going into diapause (Niva and Takeda 2002, 2003). In studies that focused more explicitly on determining the critical photoperiod that induces diapause, results have also been inconsistent. In one study, 15L:9D at 25°C caused 40% of individuals to go into diapause and 100% with 14h 45min light or less (Yanagi and Hagihara 1980). Conversely, in another study 14L:10D at 25°C did not induce diapause, but at 13L:11D, 9 out of 10 individuals went into diapause (Watanabe 1979). All the aforementioned studies use ovary development to determine diapause status, with lack of visible development indicating diapause.

Across pentatomid species, the life stage sensitive to diapause-inducing cues varies. It has been found to range from second instar to adult, but is generally the stage that immediately precedes the diapausing stage (Saulich and Musolin 2014). Watanabe (1979) attempted to determine if a specific *H. halys* life stage was sensitive to photoperiodic shifts from 16L:8D to 12L:12D, or the reverse. They found that rearing at a constant 25°C, 12L:12D from egg to adult or from first instar to adult resulted in no developed ovaries. However, a switch from 12L:12D to 16L:8D after egg hatch or adult

eclosion resulted in a mix of diapausing and non-diapausing individuals. Similarly, when the pattern was reversed and individuals were taken from 16L:8D to 12L:12D after adult eclosion, only a portion of individuals entered diapause. They concluded that the photoperiod adults experience is important for ovary development, but immature stages are also sensitive (Watanabe 1979).

Most often, diapause enhances an insect's ability to survive cold temperatures, but diapause and cold tolerance are not always related (Denlinger 1991). Šlachta et al. (2002) found that diapause was required to achieve adequate cold tolerance to survive winter conditions in the stink bug *Graphosoma lineatum* L. For the southern green stink bug, *Nezara viridula* (L.), conflicting reports exist. Lower supercooling points appeared independent of diapause status (Elsey 1993), but diapause significantly increased the likelihood of winter survival (Musolin et al. 2010). This difference points to the possibility that other factors, besides freezing, contribute to overwintering mortality. For *H. halys*, the relationship between diapause, cold tolerance, and overwintering survival is unknown. We have previously shown that supercooling points are lowest in winter, but diapause status was not investigated (Cira et al. 2016)

Information about the triggers and effects of diapause on insects is important for building phenology models (Nielsen et al. 2016), understanding the structure and purpose of seasonal adaptations (Danks 2007), relationship to cold hardiness (Denlinger 1991), and insect abundance and distribution (Bale 2010), and for developing effective management strategies (Denlinger 2008). To date, the literature on the triggers of *H. halys* diapause is inconsistent and the effects of diapause on cold tolerance is sparse. Thus, our objectives were to: 1) find laboratory rearing conditions that induce diapause and achieve comparable cold tolerance (i.e., supercooling points and lethal temperatures) to field-acclimatized *H. halys*, and 2) explore plasticity of *H. halys* diapause through the effects of changing conditions (post-diapause induction) on feeding, supercooling points, ovary development, and lethal temperatures.

Materials and Methods

Insects

All *H. halys* originated from a laboratory colony at the University of Minnesota or from a natural field population in Wyoming, MN (45.33442 N, 92.99579 W) (described below). The colony was founded from egg masses obtained in spring of 2012 from a laboratory colony at the University of Maryland, and was supplemented with field-collected eggs from Virginia in the summer of 2013. Insects from the colony were reared in 38×38×61 cm mesh cages (BioQuip, Rancho Dominguez, CA) and provisioned with potted snap bean plants (*Phaseolus vulgaris* L. cv 'Romano Bush'), fresh organic snap bean pods, dried raw organic sunflower (*Helianthus annus* L.) and soybean seeds (*Glycine max* (L.) Merr.) *ad libitum*. These mesh cages were located either in laboratory programmable growth chambers (Percival Scientific, Perry, IA) (temperature and photoperiod details described below), or within a larger wire screen enclosure outdoors on the St. Paul campus of the University of Minnesota (44.988266 N, 93.180824 W).

Insects collected from the field in Wyoming, MN (Experiment 3) were gathered from the exterior of a residence from October 15, 2014 to November 4, 2014, and placed in circular, ventilated plastic dishes (18.5cm diameter x 8cm; Pioneer Plastics, Inc., North Dixon, KY) with a 25×89 cm piece of cotton canvas, and provisioned with dry organic soybean seeds. The dish remained outdoors on a covered porch in Wyoming, MN until the date of testing when they were transferred from Wyoming to St. Paul, MN in a cooler.

Determination of instars was based on morphological traits outlined in Hoebeke and Carter (2003) and determination of sex was based on visual inspection of the ventral, apical section of the abdomen (Rice et al. 2014). Diapause status was determined by dissecting females under 8x magnification to measure ovary development according to Watanabe et al. (1978). Ovaries from the IV stage of development (i.e., at least one fully formed oocyte) onward were considered developed.

Statistics

All analyses were conducted in R version 3.4.0 (R Core Team 2017) and RStudio Desktop version 1.0.136 (RStudio Team 2016). All plots were constructed using R (packages and *commands*: ggplot2, *ggplot,* (Wickham 2009), ggthemes various *scale_*

commands (Arnold 2017), gridExtra *grid.arrange* (Auguie and Antonov 2016), Rmisc, *summarySE* (Hope 2013)). When linear regression was used, backwards elimination (i.e., stepwise removal of non-significant model terms ($p > 0.05$) from most to least complex) was used to determine final model parameters unless otherwise noted. Each iteration of a model was tested for assumptions of normality and heteroscedasticity via a Shapiro-Wilk normality test and Breusch-Pagan test (car, *ncvTest* (Fox and Weisberg 2011a)), respectively. If either test did not meet assumptions ($p < 0.01$) data were transformed using a Box-Cox power transformation (MASS *boxcox* (Ripley and Venables 2002)). Where applicable, means were compared with a Tukey's honest significant differences multiple comparisons test.

Experiment 1a: Diapause induction

To determine laboratory rearing conditions that induce diapause, *H. halys* were reared at one of three rearing conditions (see Table 2.1 for details). Note that, while we did not know beforehand which treatments would or would not results in diapause induction, we refer to treatments based on their hypothesized effects (e.g., diapausing, non-diapausing) in the tables and descriptions for simplicity. As adults eclosed in these conditions they were separated into cohorts of similarly aged individuals (i.e., eclosion no greater than 7 days from another individual in the cohort) to have an approximate age at testing. We measured the effects of rearing condition and adult age on diapause induction. Individual insects were considered the replicated unit. A 3-sample Pearson's χ^2 test for proportions was used to test the effect of three adult ages on the proportion of *non-diapausing (lab)* females with developed ovaries (Table 2.4). No *diapausing (lab)* females had developed ovaries, so no statistics were run for this group of individuals (Table 2.5).

Experiment 1b: Combining treatments

To determine if different sexes and adult ages of *non-diapausing (lab)* adults could be combined for further analysis we compared the mean supercooling points of these groups. Linear regression was used to test the effect of sex, adult age, and the interaction of the two on the supercooling points of *non-diapausing (lab)* adults (Table 2.4).

To determine if different photoperiods and adult age of *diapausing (lab)* adults could be combined for further analysis we compared the mean supercooling points of these groups. The effect of sex was not tested due to low sample sizes when sexes were split. Linear regression was used to test the effect of photoperiod, adult age, and the interaction of the two on the supercooling points of *diapausing (lab)* adults (Table 2.5).

Here, and in all following experiments, supercooling points were measured using contact thermocouple thermometry; individual adults were placed in close proximity to coiled copper-constantan thermocouples (e.g., Hanson and Venette 2013) that were attached to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Temperatures were recorded once per second and logged using Tracer-DAQ software (Measurement Computing, Norton, MA). If an exotherm, or spontaneous release of heat indicative of a phase change from liquid to solid, was observed, the lowest temperature reached before the exotherm was recorded as an individual's supercooling point (Lee 1991).

In 2013, the insect and thermocouple were confined in a 20 or 35ml syringe (Monoject syringes with leur lock tip), per Hanson and Venette (2013), that was placed at the center of a 20 x 20 x 20cm polystyrene cube and then into a -80°C freezer according to Carrillo et al. (2004). The insects cooled at a realized rate of ~ -0.82ºC ± 0.008°C per minute. In 2014-15, the insect and thermocouple were confined in an 18x150mm (ODxL) Kimax glass test tube, stabilized with one sheet (11.18 x 21.34cm) of Kimtech delicate task wipers, and a rubber test tube stopper with a 5mm hole. This apparatus was placed in a refrigerated bath of silicon 180 oil (Thermo Fischer Scientific A40, Waltham MA) at room temperature and chilled at a rate of ~ -0.95ºC ± 0.003°C per minute.

Experiment 1c: Comparison of laboratory- and field-induction of diapause

To assess how similar laboratory-induction of diapause was to field-induction of diapause we compared mean supercooling points and mass of adult *H. halys,* and ovary development of females, reared in laboratory or field diapause-inducing conditions and a laboratory non-diapausing control (see Table 2.1 for details). Mass was measured immediately preceding cooling (precision = 0.0001). First, to determine if the data from experiments conducted in two separate years could be compared, linear regression was

used to test for significant differences between the controls in each year. To test the effect of experiment year on mass, linear regression models were made for each sex separately because females are known to weigh more than males (Lee and Leskey 2015). No significant differences of supercooling points or mass of either sex were found based on experiment year, so the experiments were pooled for subsequent analysis.

Combining treatments where appropriate from Experiment 1a and 1b, we used linear regression to test the effects of diapause status, sex, and the interaction of the two on supercooling points (Table 2.6). To test the effect of diapause status on mass, models were made for each sex separately. No adequate transformation was found for female masses, so a Kruskal-Wallis rank sum test was used, followed by Dunn's test with a Holm's multiple comparisons adjusted α (α = 0.05) (dunn.test, *dunn.test* (Dinno 2017)). Male masses did not need to be transformed and linear regression was used. A 2-sample Pearson's χ^2 test for proportions was used to test the effect of diapause status on ovary development between *non-diapausing (lab)* and *diapausing (lab)* females.

Experiment 2: Variation of cold tolerance and feeding following diapause induction

To better understand how cold tolerance and feeding might change following diapause induction we reared insects in diapause inducing conditions (20°C 12L:12D) until they reached adulthood. Newly eclosed adults were individually transferred every Monday, Wednesday, and Friday to a lidded plastic cup (Translucent 473 ml, Consolidated Plastics Stow, OH) and randomly assigned to one of three adult maintenance conditions (see Table 2.2 for details), and one of three lengths of time at those conditions (7, 14, or 21 days). Individuals were provisioned with three dry organic soybean seeds and a cotton ball soaked in water. Cotton balls were re-wetted as needed. Soybean seeds were removed and replaced periodically; removed seeds were saved for feeding analysis (see below).

Lethal temperatures

Individuals in this experiment were randomly assigned to one of sixteen exposure temperatures (every 1°C from -5 to -20°C) or a room temperature control (handled in the same way except for exposure to cold temperatures). After remaining at their adult maintenance conditions for the assigned length of time, insects were cooled in a chiller

bath, as described above, until they reached the assigned exposure temperature. Then they were immediately removed from the chiller bath to warm to room temperature. After warming, insects were transferred to individual plastic cups, provisioned with soybean seeds and water, and placed at 25°C 16L:8D. After 24h, they were checked for survival. Mortality was defined as a complete lack of movement or an inability to walk after being gently prodded with a soft-bristle paintbrush.

To test the effect of exposure temperature, maintenance conditions, length of time at maintenance conditions, and all interactions on proportion mortality, a generalized linear model with a binomial logit link function was used. Model parameters were determined through modified backward elimination (i.e**.,** step-wise removal of non-significant terms ($p > 0.05$) starting with the term with the highest p-value) (Fig. 2.1).

Supercooling points

Some individuals in this experiment began to freeze before reaching their assigned exposure temperature. To test the effect of exposure temperature, maintenance conditions, length of time at maintenance conditions, and the interaction of maintenance conditions and length of time at maintenance conditions on the proportion of insects that reached their supercooling point, a generalized linear model with a binomial logit link function was used. Model parameters were determined through modified backwards elimination (i.e., step-wise removal of non-significant terms ($p > 0.05$) starting with the term with the highest p-value). A Tukey's honest significant difference test was used to compare means (multcomp, *cld, glht* (Hothorn et al. 2008)) (Fig. 2.2).

Feeding

To test whether diapausing individuals feed, and if so, what the variability of feeding was, we collected soybean seeds from a subset of individuals (n=166) in this experiment. Following the methods of Bowling (1979) seeds were stained to count salivary flanges. Briefly, seeds were immersed in an acid fuchsin staining solution for approximately two minutes and then rinsed gently with water. After air-drying on filter paper, stained salivary flanges on each seed were counted under 8× magnification. The number of feeding flanges/day was calculated by dividing the total number of feeding flanges an individual produced by the number of days since adult eclosion.

Firth's bias-reduced penalized-likelihood logistic regression with binomial error distribution was used to test the effect of maintenance conditions, length of time at maintenance conditions, and the interaction of the two on the *proportion of individuals which fed* (logistf, *logistf, backward* (Ploner 2016)). This method can tolerate complete separation (Firth 1993, Heinze and Ploner 2004). A Tukey's honest significant difference test was used to compare means (multcomp, *cld, glht* (Hothorn et al. 2008)) (Table 2.7). Linear regression was used to test the effect of maintenance conditions, length of time at maintenance conditions, and their interaction on the *number of feeding flanges produced per day*. Individuals that did not feed (i.e., produced zero flanges) were not included in this analysis (Table 2.7).

Experiment 3 & 4: Survival after long-term cold exposure

To better understand the effects of long-term cold exposure on diapausing and non-diapausing adult *H. halys*, we transferred insects in October to one of two locations to mimic overwintering habitats. Adults were placed into circular plastic dishes (as described above) either in: 1) an unheated shed (44.98908 N, 93.18628 W) mimicking a cold overwintering microhabitat, or 2) a walk-in cooler (mean temperature ± SEM, 4.52°C ± 0.001 with constant darkness) to mimic a cool but not cold overwintering microhabitat. Groups of approximately 20 individuals were pulled from these locations every month starting in December and assessment of mortality took place in the walk-in cooler so individuals did not warm excessively before testing. Mortality was defined as a complete lack of movement after being gently prodded with a soft-bristle paintbrush, or complete desiccation of the insect's body. Living individuals were then cooled using the aforementioned methods to measure their supercooling points. Additionally, adults were collected from Wyoming, MN (as described above) and tested in October and November 2014.

Linear regression was used to test the effect of sex, testing month, and their interaction on supercooling points of *diapausing (field)* adults from St. Paul and Wyoming, MN. No adequate transformation was found for supercooling points, so a Kruskal-Wallis rank sum test was used, followed by Dunn's test with a Holm's multiple comparisons adjusted α (α = 0.05) (dunn.test, *dunn.test* (Dinno 2017)). To test the effect

of testing month on adult mass, linear regression was also used, but models were made for each sex separately (Table 2.8). High mortality of *non-diapausing (lab)* adults after long-term cold storage prohibited statistical comparison (Table 2.9).

Comparison of lethal temperatures between laboratory- and field-induction of diapause

The lethal temperatures of three groups of adult *H. halys*, diapausing (lab) and (field), and non-diapausing (lab), were modeled and compared to assess how similar laboratory-induction of diapause was to field-induction of diapause (see Table 2.3 for details). Diapausing (lab) individuals were those from Experiment 2. Non-diapausing (lab) adult *H. halys* in October 2013 and diapausing (field) individuals in December 2013 and 2014 were randomly assigned to one of four temperature treatments (-5, -10, -15, -20°C) or a room temperature control (handled in the same way except for exposure to cold temperatures). They were cooled and assessed according to the aforementioned methods. A generalized linear model with a binomial logit link function was used followed by a Tukey's honest significant difference test (multcomp, *cld, glht* (Hothorn et al. 2008)) (Fig. 2.3).

Results

Experiment 1a: Diapause induction

The proportion of *non-diapausing (lab)* females with developed ovaries was statistically the same across ages ($\chi^2 = 0.88$, $p = 0.65$), and no *diapausing (lab)* females had developed ovaries.

Experiment 1b: Combining treatments

Adult age ($F_{2, 61} = 3.14$, $p = 0.05$), sex ($F_{1, 60} = 0.19$, $p = 0.66$), and the interaction of sex and adult age ($F_{2, 58} = 0.59$, $p = 0.56$) did not significantly affect supercooling points of *non-diapausing (lab)* individuals, so sexes and ages were pooled for subsequent analysis (Table 2.4). Supercooling points of *diapausing (lab)* individuals were not significantly affected by photoperiod ($F_{1, 60} = 2.43$, $p = 0.12$), or the interaction of photoperiod and adult age ($F_{1, 59} = 0.001$, $p = 0.97$), so photoperiods were pooled for subsequent analysis. Adult age significantly affected the supercooling points of

diapausing (lab) individuals ($F_{1, 61} = 24.67$, $p < 0.0001$), where older adults had significantly lower supercooling points (Table 2.5).

Experiment 1c: Comparison of laboratory- and field-induction of diapause

Sex ($F_{1, 180} = 1.52$, $p = 0.22$), and the interaction of diapause status and sex ($F_{3, 177} = 0.21$, $p = 0.89$) did not significantly affect supercooling points, but diapause status did ($F_{3, 181} = 34.88$, $p < 0.0001$). Compared to *non-diapausing (lab)* adults a significant reduction in mean supercooling points was found for both *diapausing (field)* adults and *diapausing (lab)* adults that were at least 34d from adult eclosion (Table 2.6). Supercooling points of individuals less than 34d old in the diapausing (lab) group did not differ from non-diapausing (lab) individuals (Table 2.6). The mean ± SEM proportion of *non-diapausing (lab)* females with developed ovaries was 0.82 ± 0.07 (n=28), and was significantly higher ($\chi^2 = 39.22$, $p < 0.0001$) than the proportion of *diapausing (lab)* females with developed ovaries (0.00 ± 0.00 (n=32)). Diapause status had a significant effect on *H. halys* mass for both females ($\chi^2 = 53.19$, $p < 0.0001$) and males ($F_{3,115} = 29.33$, $p < 0.0001$) (Table 2.6). We did not find a consistent pattern of increasing or decreasing mass between sexes, however the heaviest individuals for both sexes were those that had acclimatized in the field (Table 2.6).

Experiment 2: Variation of cold tolerance and feeding following diapause induction

Changes to the adult maintenance conditions, post-diapause induction, did not influence the proportion of females with developed ovaries. No females from the colder (10°C 12L:12D, n=33), constant (20°C 12L:12D, n=31), or warmer (25°C 16L:8D, n=30) treatments had developed ovaries or laid any eggs. All individuals in this experiment were in diapause for the entirety of the experiment.

Lethal temperatures

Maintenance condition, length of time at maintenance conditions, and their interaction did not significantly affected mortality at a given temperature (Fig. 2.1A, B, & C), so individuals from all maintenance conditions, and lengths of time at those conditions were pooled. As exposure temperature decreased, mortality increased (Fig 2.1A, B, & C). Very little control mortality occurred (proportion survival ± SEM, 0.99 ± 0.01 (n=83)).

Supercooling points

Exposure temperature ($\chi^2 = 121.81$, $p < 0.0001$), maintenance conditions ($\chi^2 = 12.80$, $p = 0.002$), length of time at maintenance conditions ($\chi^2 = 10.37$, $p = 0.006$), and the interaction of exposure temperature and maintenance conditions ($\chi^2 = 6.57$, $p = 0.04$) significantly affected the supercooling point distribution of diapausing adults. Individuals reared and maintained at a constant 20°C 12L:12D had significantly lower supercooling points than individuals reared at 20°C 12L:12D but moved to a colder temperature (10°C) as adults. However, supercooling points of individuals reared at 20°C 12L:12D and moved to a warmer temperature (25°C) and longer photoperiod (16L:8D) as adults did not significantly differ from either maintenance regime (Fig. 2.2A). Across maintenance conditions, adults that were 7 and 21 days old reached their supercooling points at significantly warmer temperatures than individuals 14 days from eclosion (Fig. 2.2B).

Feeding

Maintenance condition significantly affected the proportion of diapausing adults that fed ($p < 0.0001$) (Table 2.7), but length of time at maintenance conditions ($p = 0.13$) and their interaction did not ($p = 0.92$). Maintenance condition ($F_{2, 110} = 30.22$, $p < 0.0001$) and length of time at maintenance conditions ($F_{2, 110} = 4.11$, $p = 0.02$) significantly affected the mean number of flanges/individual/day of individuals which fed at least once but their interaction did not ($F_{4, 106} = 2.10$, $p = 0.09$) (Table 2.7). Adults in the maintenance conditions for one week produced a mean ± SEM number of flanges/day of1.61 ± 0.17 (n=42) which was significantly more than the number of feeding flanges/day adults in the maintenance conditions for three weeks produced (0.90 ± 0.09 (n=38)), but not significantly different from the number of flanges/day produced by adults in the maintenance conditions for two weeks (1.12 ± 0.13 (n=35)). The mean total flanges ± SEM per individual was: 11.24 ± 1.22 by 7d, 15.74 ± 1.80 by 14d, and 19.00 ± 1.96 by 21d. There was a decreased rate of feeding over time; 60% decrease in quantity of feeding over two weeks compared to one week, and 79% decrease in quantity of feeding over three weeks compared to two.

Experiment 3 & 4: Long-term field acclimatization

For *diapausing (field)* individuals from St. Paul and Wyoming, MN, the date of testing ($F_{1, 51} = 27.71$, < 0.0001) significantly affected supercooling points but sex ($F_{1, 50} = 0.03$, $p = 0.87$), and their interaction ($F_{1, 49} = 2.31$, $p = 0.13$) did not (Table 2.8). Supercooling points in October were higher than all other months. Sex ($F_{1, 51} = 89.02$, $p < 0.0001$) significantly affected mass but the date of testing ($F_{1, 50} = 0.32$, $p = 0.57$), and their interaction ($F_{1, 49} = 0.44$, $p = 0.51$) did not (Table 2.8). Masses did not steadily increase or decrease through time, but females were heavier than males. *Non-diapausing (field)* individuals were largely unable to survive winter temperatures in an unheated shed in Minnesota, or at a low but not freezing temperature (4.52 °C) in a walk-in cool room (Table 2.9). No developed ovaries were found at any date of testing for *diapausing (field)* females, but *non-diapausing (field)* females did have developed ovaries.

Comparing lower lethal temperatures between laboratory-induction and field-induction of diapause

Exposure temperature ($\chi^2 = 198.31$, $p < 0.0001$) and diapause status ($\chi^2 = 22.95$, $p < 0.0001$) significantly affected the lower lethal temperature of adults but their interaction did not ($\chi^2 = 2.57$, $p = 0.26$). The method of diapause induction – laboratory-induced or field-induced – did not affect lower lethal temperatures (Fig. 2.3). Both diapausing populations could survive significantly lower temperatures than non-diapausing (lab) individuals (Fig. 2.3). No control mortality of diapausing (field) individuals was observed (n=17) in 2013 but was 0.06 ± 0.06 (n=17) in 2014. Non-diapausing (lab) control mortality was 0.12 ± 0.08 (n=17).

Discussion

Understanding *H. halys* diapause induction and dynamics is important for management of this pest. Here, we report laboratory rearing conditions that induce diapause and achieve comparable cold tolerance (i.e., supercooling points and lethal temperatures) as field-induction of diapause causes, and measure potential variation of supercooling points, lethal temperatures, mass, feeding, and overwintering survival of diapausing *H. halys.*

Diapause

We determined laboratory rearing conditions which induce diapause and result in statistically similar cold tolerance (i.e., supercooling points and lower lethal temperatures) as field-acclimated individuals (Table 2.6, Fig 2.3). We did not attempt to determine the exact threshold of diapause-inducing or diapause-breaking cues, or which specific cues (i.e., temperature, light, or an interaction of the two) are the predominant trigger(s) to enter or break diapause. Rearing regimes of 20°C 12L:12D and 20°C 8L:16D initiate *H. halys* diapause (Table 2.4), while a 25°C 16L:8D regime did not (Table 2.5). Additionally, we found diapause allowed for survival at significantly lower temperatures than a non-diapausing state (Fig. 2.3), and was necessary for survival after long-term exposure to cold temperatures (Table 2.8, 2.9).

Following diapause-induction, adults maintained at a longer photoperiod (16L:8D) and higher temperature (25°C) did not resume normal reproductive development after three weeks (~227 DD). Additionally, we found that no ovarian development occurred from October to March when maintained at 4.52°C and constant darkness (Table 2.8). Previous research has shown that a minimum of 7 weeks at 25°C was needed before reproduction resumed after diapause induction (Taylor et al. 2017).

In laboratory and field non-diapausing regimes, we consistently found, regardless of adult age, a proportion of females that did not appear to have developed ovaries (Tables 2.4 and 2.9). It is possible that our dissection method may result in false negatives, perhaps when dissection closely follows an oviposition event. In previous *H. halys* work, this dissection method was often employed and, if inherently variable, may explain some of the contradictory results found in the literature. However, in Experiment 2, we individually monitored females for oviposition, which never occurred, so feel confident that lack of ovary development, when found in all females, is still a reliable proxy for determining diapause status.

Supercooling points

An increased capacity to supercool greatly enhances arthropod cold tolerance (Tauber et al. 1986). It has previously been shown that *H. halys* supercooling points

change based on sex, season, and acclimation location (Cira et al. 2016). In the present study, we found that supercooling points of diapausing *H. halys* decrease over time (Table 2.5, 2.8), eventually to a significantly lower temperature than non-diapausing *H. halys* (Table 2.6). Therefore, diapause is another factor affecting *H. halys* supercooling points.

Between Experiment 1 and 2 we did not see the same pattern of decreasing supercooling points over time (Table 2.5 vs. Fig. 2.2B). In Experiment 2, we found regardless of adult maintenance conditions, diapausing adults 14 days from eclosion had significantly lower supercooling points than those 7 or 21 days from eclosion (Fig. 2.2B). Whereas in Experiments 1 and 3, over longer time periods, supercooling points only decreased through time. It is possible that the design of these experiments differed in a way that led to these different results. Specifically, in in experiment 2 measurements of individuals were taken within the first three weeks after adult eclosion, whereas in experiment 1 and 3 we started measuring supercooling points only after three weeks had passed, and over a greater length of time.

Pre-diapause conditions can affect the successful induction and the full intensification of diapause (Tauber et al. 1986), and for some arthropods, the degree of response to changing conditions scales with changes to photoperiod or temperature (Danks 2007). In our study, supercooling points and ovary development did not scale with decreasing photoperiod (Table 2.5). We did find, in Experiment 2, supercooling points following diapause-induction, varied depending on maintenance conditions (Fig. 2.2A). Plasticity in supercooling points dependent on maternal rearing regime was found for diapausing blow flies but not non-diapausing blow flies (Coleman et al. 2014). Many factors affect supercooling points such as accumulation of antifreeze proteins, low-molecular-weight cryoprotectants (Lee 1991). Feeding has a multidimensional relationship with supercooling. On one hand, feeding is necessary to accumulate antifreeze proteins, low-molecular-weight cryoprotectants that lower supercooling points (Lee 1991). While on the other hand, food in the gut offers potential ice nucleation cites which could lead to higher supercooling points (Salt 1958a). We measured the proportion of adult *H. halys* feeding and quantity of salivary flanges in the current study, and found

maintenance conditions significantly affected both measures and adult age significantly affected salivary flange quantity. Therefore, we cautiously interpret plastic responses of supercooling point data, knowing our study was not designed to resolve the potential confounding role temperature has on feeding and subsequent ability to synthesize antifreeze proteins or accumulate low-molecular-weight cryoprotectants. Future studies could consider measuring the ability of *H. halys* to produce antifreeze proteins and/or low-molecular-weight cryoprotectants at different temperatures.

Lethal temperatures

A reliable indicator that an insect has progressed from the pre-diapause to diapause state is their survival rate at low temperatures (Saulich and Musolin 2014). The depth, or intensity of diapause is a relative measure of change, including for example, the level of resistance to extreme temperatures (Tauber et al. 1986). While supercooling points can indicate diapause intensity and/or a level of cold tolerance, they are not a precise predictor of mortality for *H. halys*. Being chill-intolerant, *H. halys* dies before reaching their supercooling points (Cira et al. 2016). Thus, for this species, it is important to measure lethal temperatures in addition to supercooling points. No plasticity of lower lethal temperatures for diapausing *H. halys* was found between different maintenance conditions and lengths of time at those maintenance conditions (Fig. 2.1A, B, C). The lethal temperatures for diapausing adults of different ages maintained in different maintenance conditions, were the same. This lack of plasticity could be advantageous to deal with fluctuating autumn temperatures. Plasticity of lower lethal temperatures has been found in other insects, such as non-diapausing spotted wing drosophila (Jakobs et al. 2015) and some species of collembola (Slabber et al. 2007).

Lower lethal temperatures of diapausing (lab and field) *H. halys* significantly differed from non-diapausing (lab) *H. halys* (Fig. 2.3). Finding no significant difference in lower lethal temperatures between field- and laboratory-diapausing individuals is promising for extrapolating results of laboratory studies to field settings. Equivalency between laboratory and field rearing is advantageous for future studies that would like to use laboratory colonies in cold tolerance studies, for greater uniformity and control of tested individuals, or to have access to diapausing individuals year-round. From this

comparison, we also see that induction of *H. halys* diapause immediately confers a greater ability to survive cold temperatures.

Mass

In addition to significantly affecting supercooling points, diapause had a significant effect on *H. halys* mass (Table 2.6). The reason for this significant mass difference is not due to available food, as all insects in this experiment had access to the same type of food. Lee and Leskey (2015) also found overwintering females were heavier than non-overwintering females. However, unlike in our study, male masses did not change between overwintering and non-overwintering (Lee and Leskey 2015). This difference could have arisen because in Lee and Leskey (2015) field collected individuals' diet was not standardized. We also compared the masses of overwintering *H. halys* through the winter.

Feeding

Diapausing *H. halys* fed, though the conditions at which they were maintained significantly affected the proportion of adults that fed and the quantity of feeding flanges (Table 2.7). Pentatomidae salivary flanges and sheaths have been shown to be predictive of crop injury and/or damage (Bowling 1979, 1980, Viator et al. 1983, Barbour et al. 1990, Bundy et al. 2000, Zeilinger et al. 2015), and *H. halys* feeding sites have been correlated with feeding injury that results in impacts on crop yields and quality (Leskey et al. 2012b, Owens et al. 2013, Cissel et al. 2015). However, salivary flanges should not be used to measure quantity of consumption or preference (Zeilinger et al. 2015). We therefore view our results only in the context of crop injury. We assume the salivary flanges measured in the laboratory will correlate to crop injury and lead to crop damage (i.e., loss in host utility (Pedigo et al. 1986)).

From week-to-week, the mean total flanges per diapausing adult increased at a rate less than what would be expected if feeding were occurring at the same rate across weeks. As diapausing adult *H. halys* aged, they produced fewer flanges. It is possible that, as diapause intensity increases, this decrease in feeding continues to the point of a complete cessation of feeding, but we did not measure feeding beyond three weeks at 25°C (227 DD). It is also possible that, like other stink bug species (e.g., *Euschistus*

heros (Fabr.) (Mourão and Panizzi 2002)), feeding decreases with age regardless of diapause. The proportion of adults which fed (all ages) significantly decreased at 10°C 12L:12D compared to 20°C 12L:12D and 25°C 16L:8D. The mean number of flanges produced by individuals that fed significantly increased in each increasing temperature regime, the most being found at 25°C 16L:8D (Table 2.7). Wiman et al. (2014) found that temperature significantly affected probing (i.e., any contact of the proboscis to a food source) in adult *H. halys*, though in their study, probing decreased on either side of an optimum temperature range of 16-17°C. Their study was conducted on non-diapausing *H. halys* and the metric they used (probing) was different than what we used (salivary flanges). Wiman et al. (2014) reported that a probe could last anywhere between 1 second to 185 minutes, and included non-piercing contact with the food source. Salivary flanges thus do not equate to probes, and would account for only a portion of probes.

Our study shows that diapausing *H. halys* still feed and the conditions they experience (i.e., photoperiod and temperature) affect the proportion of individuals which feed and the quantity of feeding per individual. This is important information for growers managing *H. halys* anywhere where it may enter a reproductive diapause. Even if individuals have been cued to enter diapause, they will not cease feeding within the first three weeks as an adult. Feeding status is not a reliable indicator of whether diapause has been initiated.

In summary, our study provides a foundation for future *H. halys* diapause and cold tolerance studies to rear diapausing individuals in the laboratory, which exhibit the same cold tolerance profile (i.e., supercooling points and lower lethal temperatures) as field diapausing *H. halys*. We also found that diapause increases overwintering survival, reduces supercooling points, though not immediately upon reaching adulthood, reduces lethal temperatures uniformly regardless of variable maintenance conditions and lengths of time at those conditions following diapause induction, and does not prevent feeding. This information can contribute to more precisely parameterized phenology models, predictions of overwintering mortality, understanding of feeding and potential crop injury, species distribution, and development of novel management strategies.

Tables and figures

Table 2.1. Rearing conditions used in Experiment 1a, b, and c. This experiment sought to test the effects of rearing regime and adult age on supercooling points, diapause, and mass, and compared the effects of laboratory-acclimation versus field-acclimatization. Two years of experiments were combined, see statistical methods for justification. Numbers in parentheses in the testing month column indicate sample size of treated individuals.

Rearing conditions	Egg	1st	2nd-5th	Adult	Testing month (n)
Non-diapausing (lab)	25°C 16L:8D				Oct 2013 (60) March 2014 (64)
Diapausing (lab 12L:12D)	25°C 16L:8D		20°C 12L:12D		April 2014 (27)
Diapausing (lab 8L:16D)	25°C 16L:8D		20°C 8L:16D		April 2014 (37)
Diapausing (field)*	25°C 16L:8D		Field (July 2013)		Oct 2013 (42)

*Individuals were reared in the laboratory until 2nd instars and then transferred to a field cage in St. Paul, MN

Table 2.2. Rearing conditions from Experiment 2. This experiment tested the effects of changing adult maintenance conditions, post-diapause induction, and adult age on supercooling points, diapause, lethal temperatures, and feeding. All testing occurred between June 2014 and July 2015. See materials and methods for control sample sizes.

Rearing conditions	**Egg**	**1st**	**2nd-5th**	**Adult**	**Sample size**
Colder	25°C 16L:8D		20°C 12L:12D	10°C 12L:12D	99
Constant	25°C 16L:8D		20°C 12L:12D		95
Warmer	25°C 16L:8D		20°C 12L:12D	25°C 16L:8D	102

Table 2.3. Rearing conditions from Experiment 3 and 4. These experiments compared supercooling points, mass, and diapause status over time and investigated the relationship between diapause and cold tolerance. Numbers in parentheses in the testing month column indicate sample size of treated individuals; see materials and methods for control sample sizes.

Rearing conditions	**Egg**	**1st**	**2nd-5th**	**Adult**	**Testing month (n)**
Non-diapausing (field)*	25°C 16L:8D			Field (Sep 2013)	Oct 2013 (42)
Non-diapausing (field)**	25°C 16L:8D			Field (Oct 2014)	Dec 2013-Feb 2014 (119)
Diapausing (field, St. Paul, MN)***	25°C 16L:8D		Field (July 2013)		Dec 2014-March 2015 (67)
Diapausing (field, Wyoming, MN)****	Field (2014)				Oct 2014 (19) Nov 2014 (34)

*Individuals were reared in the laboratory until adults and then transferred to a field cage in St. Paul, MN
**Individuals were reared in the laboratory until adults and then transferred to either an unheated shed or walk-in cool room
***Individuals were reared in the laboratory until 2nd instars and then transferred to a field cage in St. Paul, MN
****Individuals were collected directly from a local field population within five days of testing

Table 2.4. Supercooling points (SCP) and ovarian development of non-diapausing (lab) adult *H. halys* reared and maintained at 25°C 16L:8D (Experiment 1a and b).

Adult age (degree days*)	Mean SCP ± SEM °C (n)**	Proportion females with developed ovaries ± SEM (n)**
7-14 (75.8-151.6)	-14.71 ± 0.67 (14)	0.80 ± 0.18 (5)
14-21 (151.6-227.43)	-13.71 ± 0.31 (33)	0.88 ± 0.08 (16)
21-28 (227.43-303.2)	-12.33 ± 0.66 (17)	0.71 ± 0.17 (7)

*Degree days were calculated based on a lower developmental threshold for total development of 14.17°C (Nielsen et al. 2008a).

**Adult age did not significantly affect mean SCP ($F_{2,61} = 3.14$, $p = 0.05$) or ovary development ($\chi^2_2 = 0.88$, $p = 0.65$).

Table 2.5. Supercooling points (SCP) and ovarian development of diapausing (lab) adult *H. halys* reared and maintained at 20°C and either 12L:12D or 8L:16D. Photoperiod had no effect on SCPs so data were pooled within age. Different letters within a column indicate significant differences of means ($p < 0.05$) (Experiment 1a and b).

Adult age (degree days*)	Mean SCP ± SEM °C (n)	Proportion females with developed ovaries ± SEM (n)
20-34 (116.6-198.2)	-13.58 ± 0.63 (22) a	0.00 ± 0.00 (10)
34-41 (198.2-239.0)	-17.25 ± 0.42 (41) b	0.00 ± 0.00 (21)

*Degree days were calculated based on a lower developmental threshold for total development of 14.17°C (Nielsen et al. 2008a).

Table 2.6. Supercooling points (SCP) and mass of adult *H. halys*. Different letters within a column indicate significant differences among treatments ($p < 0.05$) (Experiment 1c).

	Mean ± SEM		
Rearing regime	**SCP °C (n)**	**Adult female mass g (n)***	**Adult male mass g (n)**
Non-diapausing (lab)	-13.41 ± 0.24 (94) a	0.1933 ± 0.0044 (58) a	0.1072 ± 0.0022 (66) a
Diapausing (lab, 20-34 days)	-13.58 ± 0.63 (22) a	0.1804 ± 0.0054 (11) b	0.1176 ± 0.0040 (12) a
Diapausing (lab, 34-41 days)	-17.25 ± 0.42 (41) b	0.1508 ± 0.0051 (21) b	0.1123 ± 0.0024 (20) a
Diapausing (field)	-16.85 ± 0.41 (28) b	0.2245 ± 0.0030 (20) c	0.1454 ± 0.0036 (21) b

* Kruskal-Wallis rank sum test, followed by Dunn's test with a Holm's multiple comparisons adjusted α.

Table 2.7. Feeding of adult *H. halys* following diapause induction. Different letters within a column indicate significant differences of means ($p < 0.05$) (Experiment 2).

Rearing conditions	Proportion of feeding individuals ± SEM (n)	Mean number of flanges/day ± SEM (n)*
10°C 12L:12D	0.24 ± 0.05 (66) a	0.43 ± 0.16 (16) a
20°C 12L:12D	0.98 ± 0.02 (53) b	1.09 ± 0.10 (52) b
25°C 16L:8D	1.00 ± 0.00 (47) b	1.66 ± 0.14 (47) c

* Means were calculated from individuals that left at least one salivary flange by the end of the experiment.

Table 2.8. Supercooling points (SCP), mass, and ovarian development of diapausing (field) adult *H. halys*. Different letters within a column indicate significant differences of means ($p < 0.05$) (Experiment 3).

	Mean ± SEM (n)			Proportion females with
Date tested *	**SCP (°C) ****	**Adult female mass (g)**	**Adult male mass (g)**	**developed ovaries ± SEM**
Oct 18, 2014	-10.51 ± 0.78 (19) a	0.1881 ± 0.0102 (10) a	0.1294 ± 0.0060 (9) ab	0.00 ± 0.00
Nov 6, 2014	-14.95 ± 0.46 (34) b	0.1880 ± 0.0051 (21) a	0.1282 ± 0.0043 (13) ab	NA
Dec 10, 2014	-16.37 ± 0.28 (19) b	0.1660 ± 0.0139 (9) ab	0.1421 ± 0.0090 (10) a	0.00 ± 0.00
Jan 10, 2015	-15.77 ± 0.49 (17) b	0.1353 ± 0.0087 (9) b	0.1062 ± 0.0050 (7) c	0.00 ± 0.00
Feb 11, 2015	-15.63 ± 0.55 (19) b	0.1707 ± 0.0065 (9) ab	0.1087 ± 0.0027 (10) b	0.00 ± 0.00
March 10, 2015	-17.17 ± 0.34 (12) b	0.1783 ± 0.0131 (4) ab	0.1099 ± 0.0049 (8) b	0.00 ± 0.00

* Individuals tested in October and November were collected as adults in the field in Wyoming, MN. In all other months, individuals were reared in the field then maintained in a cold room (4.52°C ± 0.001) from October until the testing date.

** Kruskal-Wallis rank sum test, followed by Dunn's test with a Holm's multiple comparisons adjusted α.

Table 2.9. Supercooling points (SCP), mortality, and ovarian development of non-diapausing (field) adult *H. halys*. Bugs were reared in the laboratory (25°C 16L:8D) until adulthood, then transferred to one of two field maintenance locations (Experiment 4).

Maintenance location	Date tested	Mean SCP ± SEM °C (n)	Proportion mortality ± SEM	Proportion females with developed ovaries ± SEM
Unheated shed	October 9-10, 2013	-12.58 ± 0.62 (29)	0.03 ± 0.03 (30)	NA
	Dec 10, 2014	NA	1.00 ± 0.00 (20)	0.80 ± 0.13 (10)
	Jan 10, 2015	NA	1.00 ± 0.00 (20)	0.70 ± 0.14 (10)
	Feb 11, 2015	NA	1.00 ± 0.00 (20)	0.80 ± 0.13 (10)
Walk-in cool room *	Dec 10, 2014	-15.53 ± 0.15 (2)	0.89 ± 0.07 (18)	0.80 ± 0.13 (10)
	Jan 10, 2015	NA	1.00 ± 0.00 (20)	0.70 ± 0.14 (10)
	Feb 11, 2015	NA	1.00 ± 0.00 (19)	0.26 ± 0.22 (5)

* Walk-in cool room mean temperature ± SEM, 4.52°C ± 0.001 with constant darkness.

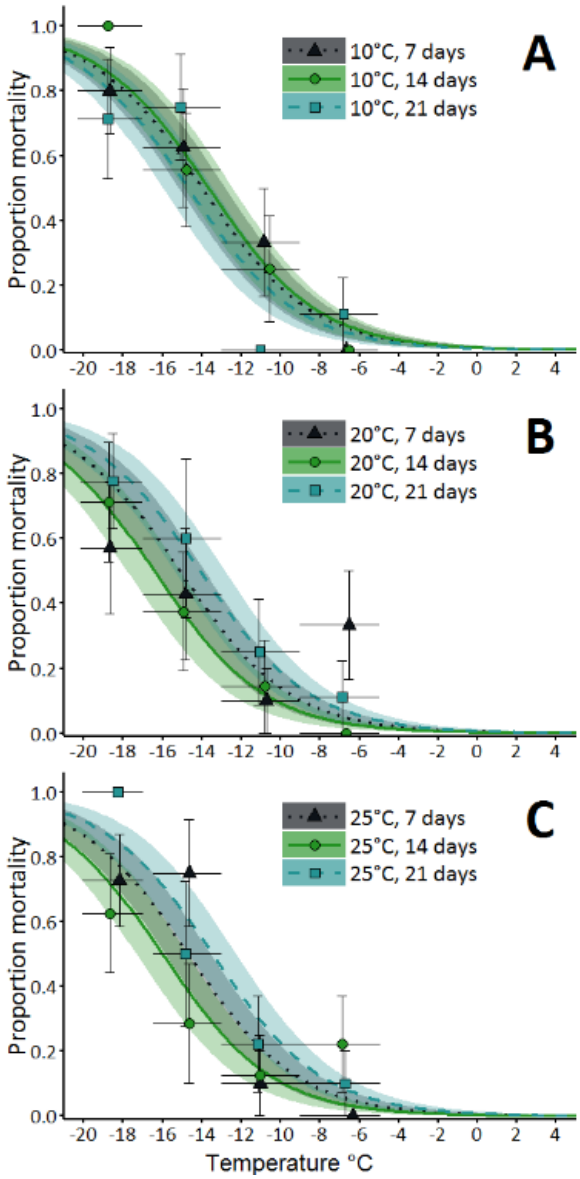

Figure 2.1. Lower lethal temperatures of adult *H. halys* following diapause induction. Individuals were reared at 20°C 12L:12D until adult eclosion, then were randomly assigned to one of three adult rearing conditions: A) 10°C 12L:12D, **B)** 20°C 12L:12D, and **C)** 25°C 16L:8D and tested at one of three adult ages. Each point represents **A)** 7-10 individuals, **B)** 5-10 individuals, and **C)** 6-11 individuals. Vertical error bars represent the SEM; horizontal error bars represent the range of binned temperatures. There were no significant differences among age groups, maintenance conditions, or their interactions (Experiment 2).

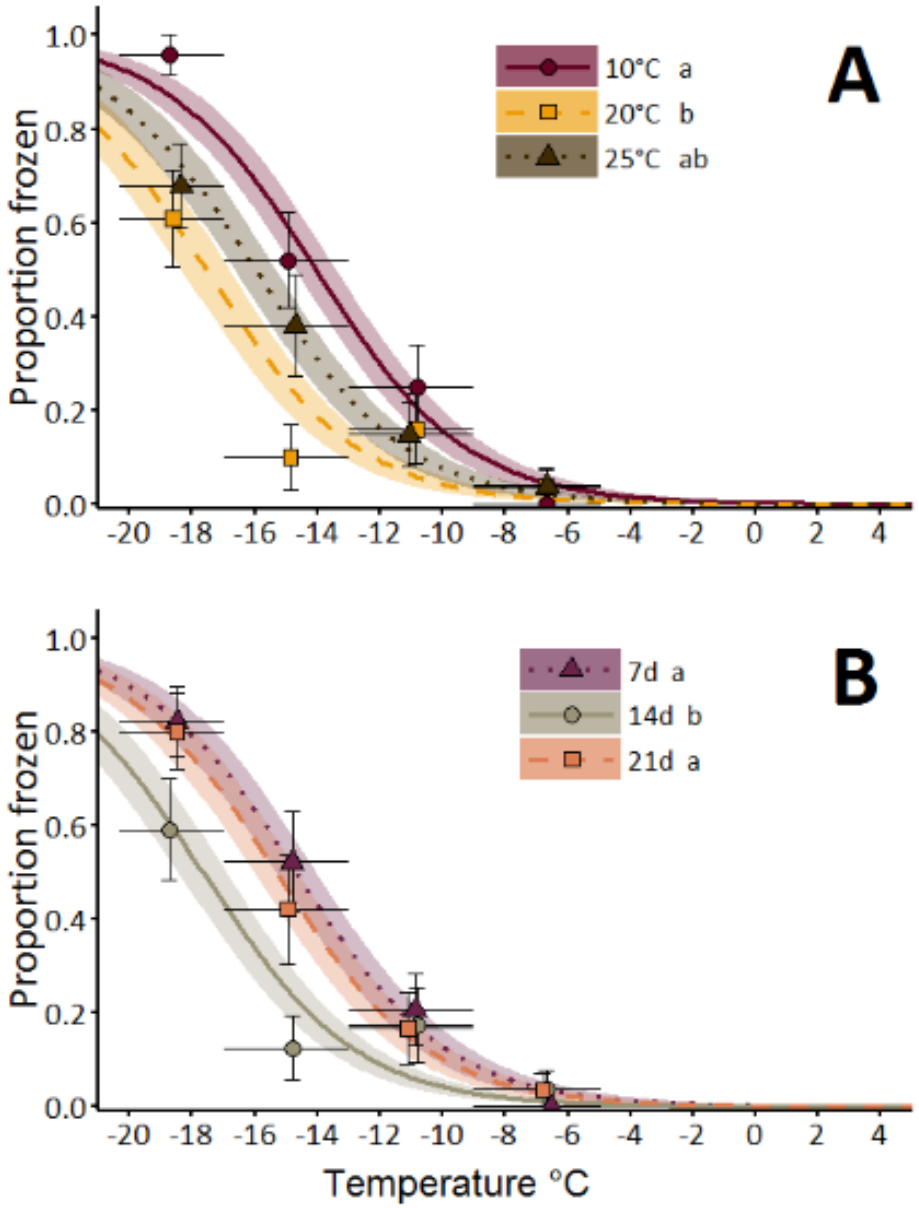

Figure 2.2. Proportion of diapausing adult *H. halys* that reached their supercooling point at a given temperature. Individuals were reared at 20°C 12L:12D until adult eclosion, then were randomly assigned to **A)** one of three adult maintenance conditions and tested at **B)** one of three adult ages. Each point represents **A)** 20-28 individuals or **B)** 19-29 individuals. Vertical error bars represent the SEM, horizontal error bars represent the range of temperatures binned. Different letters within a group indicate significant differences ($p < 0.05$) (Experiment 2).

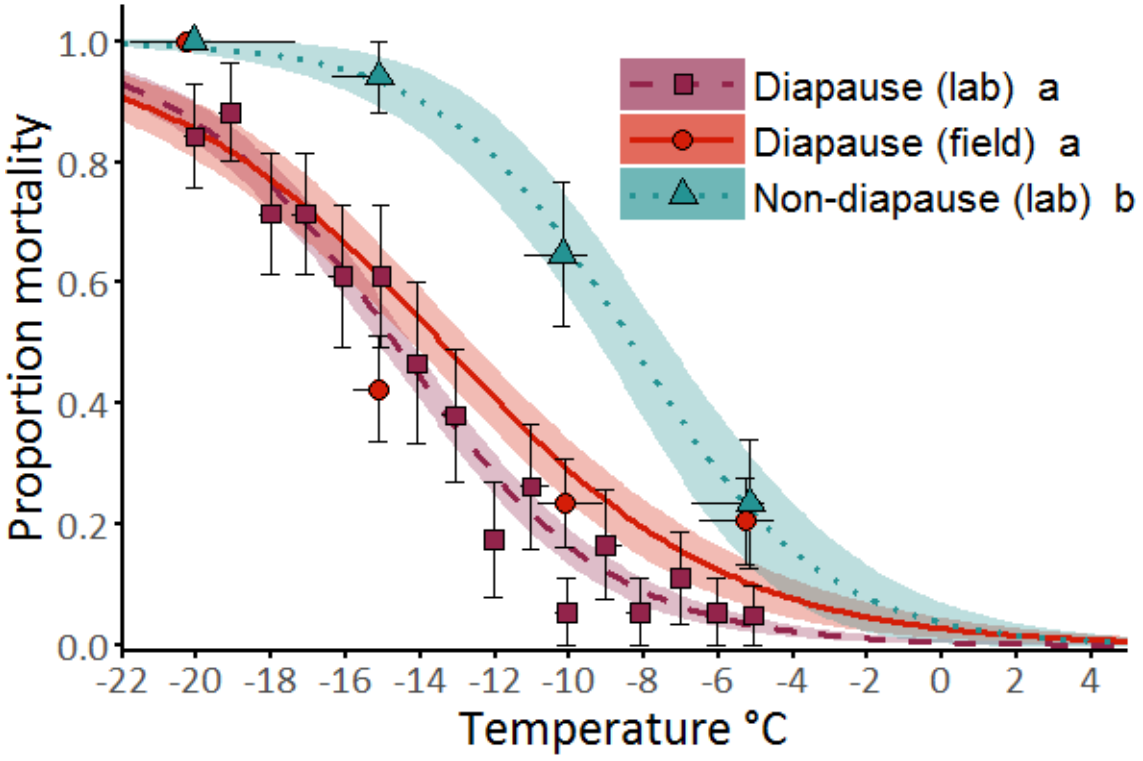

Figure 2.3. Lower lethal temperatures of adult *H. halys* following diapause induction (lab and field), or non-diapausing (lab) adult *H. halys*. Each diapause (lab) point represents 51-21 individuals, each diapause (field) point represents 34 individuals, and each non-diapausing (lab) point represents 17 individuals. Vertical error bars represent the SEM; horizontal error bars represent the range of binned temperatures.

Chapter 3: *Halyomorpha halys* mortality and sublethal feeding effects following insecticide exposure

Summary

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is a highly polyphagous invasive pest. Increased use of broad-spectrum insecticides to manage *H. halys* has resulted in secondary pest outbreaks and disruptions to integrated pest management (IPM) programs. We evaluated *H. halys* mortality, molting, and feeding after exposure to insecticides in the laboratory. Five insecticides (four active ingredients), considered less risky to natural enemies, were compared to a pyrethroid insecticide and an untreated control. Compared to the control, only azadirachtin + pyrethrins significantly reduced egg hatch, while all insecticides caused significant direct mortality to 1st and 2nd instars 5 days after hatch (DAH). Bifenthrin quickly caused complete mortality of adults, the only insecticide to statistically match this level of mortality was sulfoxaflor at 14 days after treatment (DAT). Azadirachtin + pyrethrins and sulfoxaflor significantly reduced the proportion of 1st instars that molted compared to the control. Adults that survived sulfoxaflor exposure produced significantly fewer feeding sites than the control. However, when taking into consideration both lethal and sublethal effects, all insecticides, except pyrethrins, resulted in significant reductions in feeding sites/individual compared to the control. This more complete estimate of efficacy (i.e., reduction in injury/insect), confirms the potential of several insecticides to reduce crop injury without the necessity of high direct mortality to *H. halys*.

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål), a highly polyphagous and invasive insect in North America and Europe, causes economic injury to many high-value crops such as apples, soybeans, and corn (Rice et al. 2014). Management of *H. halys* in the Mid-Atlantic region of the United States of America, where its pest status is severe, has relied mainly on chemical control since 2010 (Rice et al. 2014). Broad-spectrum insecticides such as pyrethroids, neonicotinoids, carbamates, and organophosphates are currently used against *H. halys* because they cause high, direct

mortality (e.g., Funayama 2002; Nielsen et al. 2008; Leskey et al. 2012a). However, these insecticides are also lethal to a wide range of other species, including natural enemies (e.g., Theiling and Croft 1988). The suppression of natural enemy populations through broad-spectrum insecticide applications can cause secondary pest outbreaks (Ripper 1956, Hardin et al. 1995), as has been observed after broad-spectrum insecticide use on *H. halys* (Leskey et al. 2012a, Lee et al. 2014b). Moving forward, integrated pest management (IPM) plans for *H. halys* will need to balance risks to natural enemies with reducing pest populations and crop injury (Rice et al. 2014).

Insecticide efficacy is often characterized by the amount of mortality in pest populations within a given time period (Stark and Banks 2003, Ioriatti et al. 2006, He et al. 2013, Adams et al. 2016). However, insecticides can significantly affect population dynamics through non-lethal means as well. In fact, in a review of the population-level effects of pesticides on arthropods, Stark and Banks (2003) state that characterizing the sublethal effects of insecticides, in addition to direct mortality, will produce a more accurate estimate of overall insecticide efficacy. Currently, many insecticides tested on *H. halys* do not cause a high proportion of direct adult mortality, even at the highest labeled rates (e.g., Leskey et al. 2012). High mobility of adult *H. halys* allows for quick dispersion from treated areas, which may further limit exposure to lethal doses (Lee et al. 2014b, Morrison et al. 2017b), but these insecticides may still have important sublethal impacts. Therefore, measuring sublethal effects, in addition to lethal effects of insecticides could lead to more accurate assessments of insecticide efficacy for *H. halys.*

A sublethal effect particularly pertinent to insecticide efficacy is a reduction in feeding. Salivary flanges and sheaths produced by several stink bug species have been shown to be a good predictor of crop injury and/or damage (Bowling 1979, 1980, Viator et al. 1983, Barbour et al. 1990, Bundy et al. 2000, Zeilinger et al. 2015). *Halyomorpha halys* feeding sites, in particular, have been correlated with feeding injury that results in impacts on crop yields and quality and economic losses (Leskey, Short, et al. 2012, Owens et al. 2013, Cissel et al. 2015). When *H. halys* and other phytophagous Pentatomidae feed, a viscous saliva is secreted and solidifies around the labellar lobes on the surface of the food source. This hardened saliva is called a salivary flange and

remains on the plant after the insect has stopped feeding. Inside of the food source, more viscous saliva is produced to create a hardened salivary sheath around the stylets (Chapman 2013). These dried salivary flanges and sheaths can be easily visualized for quantification by staining (Bowling 1979, 1980).

Risks to agriculturally-important natural enemies have previously been studied for certain active ingredients (AIs), including: pyrethrins, an Organic Materials Review Institute (OMRI)-certified organic insecticide, derived from *Chrysanthemum* spp. flowers; azadirachtin, also an option for organic growers in the United States, derived from the neem tree, *Azadirachta indica* A. Juss.; sulfoxaflor, a relatively new insecticide with broad-spectrum efficacy on sap-sucking insects (Zhu et al. 2011); and spinosad, a United States Environmental Protection Agency (EPA) registered "reduced risk" product, available with or without OMRI organic-certification, obtained by fermentation of the soil bacterium *Saccharopolyspora spinosad*. A reduced risk pesticide is defined by the EPA's Office of Pesticide Programs as a pesticide which poses "less risk to human health and the environment than existing conventional alternatives" (United States Environmental Protection Agency 2016). The aforementioned AIs pose less risk (i.e., direct mortality) to generalist natural enemy species (Viñuela et al. 2000, Kraiss and Cullen 2008a, 2008b, Garzón et al. 2015, Pezzini and Koch 2015, Colares et al. 2016, Tran et al. 2016, Fernández et al. 2017), and reviews of azadirachtin and spinosad concluded that both AIs displayed qualities that align well with the tenets of IPM (Schmutterer 1990, Williams et al. 2003). Conversely, bifenthrin, commonly used in many cropping systems (National Pesticide Information Center 2011) shows high toxicity in the laboratory to multiple natural enemy species, including *Harmonia axyridis* (Pallas) (Galvan et al. 2005), *Orius insidiosus* (Say), and *Encarsia citrina* Crawford (Frank 2012).

To date, research on the effects of insecticides on *H. halys* has largely focused on direct mortality with few studies characterizing sublethal effects. We were therefore motivated to conduct experiments to assess metrics that directly or indirectly contribute to reducing the potential crop injury caused by an insect after insecticide exposure. We compared the effects of six insecticides (five AIs) on *H. halys* by measuring direct mortality within an acute time period (i.e., 1-14 d), and two sublethal metrics;

development (i.e., the ability of *H. halys* to molt) and feeding (i.e., changes in quantity of feeding flanges) in the laboratory. Our aim was to obtain a more accurate estimate of overall insecticide efficacy based on lethal and sublethal metrics. In addition, by focusing on insecticidal products that could be less detrimental to natural enemy populations, our work can contribute to more sustainable IPM plans, balancing risks to natural enemies, and reductions in crop injury through lethal and sublethal effects on pest populations.

Materials and methods

Insects

Halyomorpha halys were sourced from a laboratory colony at the University of Minnesota. This colony was founded from egg masses obtained in spring of 2012 from a laboratory colony at the University of Maryland, and was supplemented with field-collected eggs from Virginia in the summer of 2013. Insects were maintained at ~25°C, 16L:8D in walk-in growth chambers. Within the growth chambers, insects were kept in 38 × 38 × 61 cm mesh cages (BioQuip, Rancho Dominguez, CA) and provisioned with potted snap bean plants (*Phaseolus vulgaris* L. cv 'Romano Bush'), fresh organic snap bean pods, dried raw organic sunflower (*Helianthus annus* L.) seeds and soybean (*Glycine max* (L.) Merr.) seeds *ad libitum*. Cages and plants were misted with water every weekday to maintain humidity. Determination of instars was based on morphological traits outlined in Hoebeke and Carter (2003) and determination of sex was based on visual inspection of the ventral, apical section of the abdomen (Rice et al. 2014).

Insecticide application

Insecticides were applied using a Teejet 8002EVS flat-fan nozzle in a motorized spray chamber. The sprayer was calibrated to deliver the equivalent of 220 L water/ha at 0.24 MPa (23.5 gallons of water/acre at 35 psi). This delivery rate optimized the area of the Petri dish covered and post-spray dry time. The spray tank was rinsed three times between treatments. Table 3.1 details the six insecticides used in our experiments. The same application rates were used to treat eggs and adults, though pyrethrins and conventional spinosad were only tested on adults. A randomized complete block design, with date of exposure as the block, was used. Within a block, the experiment was

replicated three times for eggs and six times for adults. This resulted in totals of 12 egg mass replications (295.4 ± 7.00 (SEM) eggs/treatment) and 30 adult replications/treatment. Experiments were conducted between March 25, 2013 and January 13, 2014.

Insecticide exposure

Halyomorpha halys eggs were exposed to insecticides topically. Egg masses were removed from snap bean leaves, along with a small amount of leaf tissue to which they were attached, approximately 4 d after oviposition. After removal, and before insecticide treatment, eggs that were not damaged and had developed red eye spots and a black egg burster were considered to be viable (personal observation). Any eggs that were not viable were noted and not included in the final analyses. Each egg mass averaged 24.63 ± 1.31 viable eggs. Individual egg masses were placed in the center of a Petri dish (100 × 15mm, VWR, Radnor, PA, USA) and then sprayed. After 24 h, a half-sheet of moistened filter paper (4.5 cm diameter; Fisher Scientific, Pittsburgh, PA, USA) was placed in the treated Petri dish with the eggs, and Parafilm (Bemis Inc. Neenah, WI, USA) was used to seal approximately 2/3 of the circumference of the Petri dish to maintain humidity, the paper being re-wetted when dry. Upon hatching, 1st instars were left in the treated dishes for 5 d after hatching (DAH) to avoid excessive handling, which may injure 1st instars. Hatching of eggs within a given *H. halys* egg mass is synchronized, with eggs hatching within 24 hours of each other; therefore, DAH for each egg in an egg mass was considered the same. No food or water was added at this stage; 1st instars are thought to only feed on the egg chorion or symbionts on the egg chorion (McPherson 1982, Taylor et al. 2014).

Adult *H. halys* were exposed to insecticides residually. No topical exposure was applied to this life stage because of their ability to fly out of the dish during the spray. Age of adults was not standardized: individuals were randomly assigned across insecticide treatments and the control in each block. One adult was added to each treated Petri dish (100 x 15mm) that had been previously sprayed and allowed to dry completely (approximately 1 h dry time). After 24 h of exposure to the dried residue (with no food or water) individuals were removed and placed individually in clean, lidded tubs

(Translucent 473 ml, Consolidated Plastics Stow, OH), provisioned with three dry organic soybean seeds (*Glycine max* (L.) Merr. and a cotton ball soaked in water. Cotton balls were re-wetted as needed. Soybean seeds were removed and replaced 7 and 14 d after treatment (DAT) and saved for feeding analysis (see below). An equal ratio of females to males was tested. Each individual adult was considered an independent replicate; exposure to treatments occurred in individual arenas and individuals remained separated throughout the experiment.

All blocks also included an untreated control, which consisted of individuals placed in unsprayed Petri dishes and clean tubs as described above. The Petri dishes and clean tub arenas were kept in growth chambers (Percival Scientific, Inc., Perry, Iowa) at 25°C, 16L:8D.

Direct mortality assessment

Eggs were monitored daily for hatch. At 1 DAH, the number of hatched eggs was divided by the number of total viable eggs (counted pre-treatment) to determine the proportion of unhatched eggs. For all subsequent life stages, mortality was defined by an inability to walk when gently prodded with a soft bristle brush. Assessment of 1st and 2nd instars was completed at 5 DAH. *Halyomorpha halys* has the ability to recover after an initial knock-down from insecticide treatments (Leskey et al. 2012a), therefore, even individuals that appeared dead remained in containers and were monitored for the full observation period.

Sublethal feeding assessment

Adults received no food or water while in insecticide-treated Petri dishes, so feeding quantification began 24 h after treatment. To quantify feeding of adults, an acid fuchsin solution was used to stain salivary flanges left on the collected seeds, following the methods of Bowling (1979). Briefly, seeds were immersed in staining solution for approximately two minutes and then rinsed gently with water. After air-drying on filter paper, stained salivary flanges on each seed were counted under 8× magnification. The number of feeding sites/individual insect was recorded. The number of feeding sites/day alive for each individual was calculated by dividing the number of feeding flanges in a given time period (e.g., week one, two, or the total of both weeks) by the number of days

alive. Fifteen of 210 individuals died between 7-11 DAT or 11-14 DAT, where we didn't have daily observations. We interpolated those individuals to have died at the midpoint between the days of observation.

Combined mortality and feeding metric

The number of feeding sites produced after a specific insecticide treatment was divided by the total number of individuals receiving that insecticide treatment (n=30 individuals) to give the expected mean number of feeding sites/individual. By including both living and dead individuals in the denominator, this metric incorporates direct mortality and sublethal feeding changes to produce a more complete estimate of insecticide efficacy.

Statistics

R version 3.3.2 (R Core Team 2017) and RStudio Desktop version 0.99.902 (RStudio Team 2016) were used for all statistical analyses. All plots were constructed using R (packages and *commands*: ggplot2, *ggplot*, (Wickham 2009), Rmisc, *summarySE* (Hope 2013)), except for Fig. 3.3, which was made in Sigma Plot (Systat Software Inc. n.d.). Where complete separation prohibited accurate calculation of maximum likelihood estimates, a dummy variable was added so that treatments could be compared. For example, all eggs treated with sulfoxaflor hatched, so in each sulfoxaflor block one unhatched egg was added to the raw data. These dummy variables are not presented in tables and figures. In one instance (i.e., bifenthrin 2nd instars at 5 DAH) complete separation occurred and the sample size was less than 5, in this case the treatment was not included in post-hoc comparisons. All post-hoc comparisons were conducted on the least squares means, or modeled medians.

Treated eggs

A single egg mass in a Petri dish was considered the independent experimental unit. For each egg mass, we counted the number of unhatched eggs, the number of dead nymphs (both 1st and 2nd instars) at 5 DAH, and proportion of 2nd instars at 5 DAH. The proportion of 2nd instars was calculated by dividing the number of 2nd instars by the number of living nymphs (both 1st and 2nd instars) at 5 DAH. Generalized linear mixed effects models (lme4, *glmer* (Bates et al. 2015), afex, *mixed* (Singmann et al. 2016)) with

binomial error distributions were used to test the fixed effects of insecticide on the three aforementioned variables. Block was included as a random effect in all three models. Likelihood ratio tests were used to calculate *P* values. Tukey's HSD (multcomp, *cld, glht* (Hothorn et al. 2008)) was used to determine significant differences ($\alpha = 0.05$) between treatments.

Treated adults

A single adult in a Petri dish was considered the independent experimental unit. Generalized linear mixed effects models (lme4, *glmer* (Bates et al. 2015), afex, *mixed* (Singmann et al. 2016)) with binomial error distributions were used to test the fixed effects of sex, insecticide treatment, and their interaction on mortality at 7 and 14 DAT. Block was included as a random effect in both models. Likelihood ratio tests were used to calculate *P* values and backwards elimination (stepwise removal of non-significant parameters at $P < 0.05$, starting with the most complex parameters) was used to determine the final model parameters. Tukey's HSD (multcomp, *cld, glht* (Hothorn et al. 2008)) was used to determine significant differences ($\alpha = 0.05$) between treatments.

When testing the effects of block, sex, insecticide, and their two- and three-way interactions on the number of feeding sheaths/day alive during the first week, second week, and both weeks combined, data was found to violate model assumptions. No appropriate transformations were found, so a non-parametric Kruskal-Wallis rank sum test was used. A Dunn's test (dunn.test, *dunn.test* (Dinno 2017)) with a Holm's multiple comparisons adjusted α ($\alpha = 0.05$) was used to determine significant differences between treatments.

Generalized linear mixed effects models (lme4, *glmer* (Bates et al. 2015), afex, *mixed* (Singmann et al. 2016)) with Poisson error distributions were used to test the fixed effect of insecticide treatment on the mean number of feeding sites/individual in the first week and across the first and second weeks. Block was included as a random effect in both models. Likelihood ratio tests were used to calculate *P* values. Tukey's HSD (multcomp, *cld, glht* (Hothorn et al. 2008)) was used to determine significant differences ($\alpha = 0.05$) between treatments.

Results

Treated eggs

Direct mortality

Insecticide treatment affected the proportion of unhatched eggs (χ^2 = 35.40, *df* = 4, *P* < 0.0001), though the only insecticide treatment different from the control was azadirachtin + pyrethrins, which significantly reduced egg hatch (Table 3.2). Insecticide treatments applied to eggs also affected nymphal mortality at 5 DAH (χ^2 = 734.75, *df* = 4, *P* < 0.0001). Residual exposure to all insecticides caused significantly greater early-instar mortality than the control, with organic-certified spinosad and bifenthrin treatments causing the highest degree of mortality, followed by sulfoxaflor, then azadirachtin + pyrethrins (Table 3.2).

Molting

Insecticide treatment applied to eggs also affected the proportion of surviving nymphs that molted to 2nd instar by 5 DAH (χ^2 = 212.06, *df* = 4, *P* < 0.0001); azadirachtin + pyrethrins and sulfoxaflor treatment resulted in significantly fewer 2nd instars at 5 DAH than the control (Table 3.2). While the proportion of organic-certified spinosad-treated individuals that molted to 2nd instar was numerically low, due to a low sample size of living individuals, statistically it did not differ from the control.

Treated adults

Direct mortality

Insecticide treatment (χ^2 = 95.74 , *df* = 6, *P* < 0.0001) affected mortality at 7 DAT. Insecticide treatment (χ^2 = 92.99 , *df* = 6, *P* < 0.0001) and sex (χ^2 = 5.21 , *df* = 1, *P* = 0.02) affected mortality at 14 DAT, but pooled sexes are presented in Figure 3.1. Sex was not a significant predictor at 7 DAT, and the interactions of insecticide treatment and sex were not significant predictors in either model (*P* > 0.05); sex therefore was removed from the final models. Adult mortality increased over time in all treatments except for pyrethrins and azadirachtin + pyrethrins (Fig. 3.1). These two insecticides resulted in initial knockdown, where individuals appeared dead or moribund, but subsequent assessments of mortality showed they recovered. For pyrethrins-treated individuals, this temporary knockdown was minimal; one individual at 1 h post-treatment and one

individual at one DAT were moribund, but by two DAT, all individuals were able to walk. Azadirachtin + pyrethrins-treated individuals showed a greater knockdown. At 1 h post-treatment, the proportion of adults that appeared dead or moribund was 0.66, but at 1 DAT the proportion appearing dead or moribund was only 0.33, and by 2 DAT the proportion of adults that were dead or moribund was reduced to 0.10. At 7 DAT, conventional spinosad, sulfoxaflor, and bifenthrin (Mean ± SEM: 0.43 ± 0.09, 0.53 ± 0.09, and 1.00 ± 0.00, respectively) caused significantly higher mortality than the untreated control (0.03 ± 0.03) (Fig. 3.1). By 14 DAT, the two spinosad formulations, sulfoxaflor, and bifenthrin (0.50 ± 0.09, 0.43 ± 0.09, 0.70 ± 0.09, and 1.00 ± 0.00, respectively) caused significantly higher mortality than the untreated control (0.10 ± 0.06) (Fig. 3.1).

Sublethal feeding

Insecticide treatment significantly affected the number of feeding sheaths/day alive in the first week ($\chi^2 = 26.38$, $df = 5$, $P < 0.0001$), second week ($\chi^2 = 31.49$, $df = 5$, $P < 0.0001$), and total across both weeks ($\chi^2 = 31.4$, $df = 5$, $P < 0.0001$). The median number of feeding sheaths/day alive of untreated adults in the first week was 0.50 ± 0.00, 1.50 (median ± first and third quartile), in the second week 2.00 ± 0.00, 2.50, and across both weeks 1.27 ± 0.00, 2.00 (Fig. 3.2). The only insecticide treatment which significantly reduced the median number of feeding sheaths/day alive was sulfoxaflor. In every time periods the median was 0.00 ± 0.00, 0.00 (Fig. 3.2).

Combined direct mortality and feeding

After pooling surviving and dead individuals, insecticide treatment significantly affected the mean number of feeding sites/individual in the first week ($\chi^2 = 768.43$, $df = 6$, $P < 0.0001$) and across the first and second week combined ($\chi^2 = 864.45$, $df = 6$, $P < 0.0001$). The mean number of feeding sites/individual for untreated adults averaged 6.23 ± 1.41 within the first week after exposure and 15.97 ± 2.10 within the first and second week combined (Fig. 3.3). Insecticide treatments that reduced the mean number of feeding sites/individual were azadirachtin + pyrethrins, organic and conventional spinosad, sulfoxaflor, and bifenthrin in the first week (2.47 ± 0.86, 1.50 ± 0.77, 2.33 ±

0.91, 0.03 ± 0.03, 0.00 ± 0.00, respectively) and across both weeks (9.27 ± 2.35, 10.23 ± 2.62, 12.70 ± 3.24, 0.10 ± 0.07, 0.00 ± 0.00, respectively) (Fig. 3.3).

Discussion

Foundational to IPM is the concept of the economic injury level (EIL), or "the lowest population density [of a pest] that will cause economic damage," where economic damage is the amount of injury that justifies the cost of control measures (Stern et al. 1959). Pedigo et al. (1986) outlined an EIL formula commonly used for insect pests; one of the key components in the formula is injury units/insect. They define injury as an effect, often negative, that an insect has on host physiology. By focusing on injury, as opposed to pest mortality, the EIL does not necessitate complete eradication of an insect population, or that pests must be dead in order to achieve the desired injury units/insect. Our study examined the potential effects that different insecticides could have on three metrics relevant to injury units/insect: direct mortality, molting, and quantity of feeding sites. Changes in feeding physiology and behavior are particularly important factors to consider for *H. halys*. Their feeding causes both direct crop injury (Leskey et al. 2012b, Owens et al. 2013, Cissel et al. 2015) and indirect injury, via transmission of plant pathogens, such as yeast (Brust and Rane 2013).

Similar to previous studies (Funayama 2002, 2012, Nielsen et al. 2008b, Leskey et al. 2012a, 2014, Lee et al. 2013c, Morrison et al. 2017b), we found that bifenthrin quickly caused high mortality to adult *H. halys*. The other insecticides we evaluated were unable to statistically match the level of direct mortality bifenthrin caused to adults, except for sulfoxaflor at 14 DAT (Fig. 3.1). Lee et al. (2014) observed higher mortality among adult *H. halys* when residually-exposed to pyrethrins and spinosad than our study. However, they used a pyrethrins rate labeled for ornamentals, nearly double the rate we used based on recommendations for row crops. Higher rates of product would be expected to cause greater mortality, but may be cost prohibitive in some cases.

When tested on *H. halys* nymphs, all insecticides caused significantly higher mortality than the control, at times statistically equivalent to bifenthrin (Table 3.2). This suggests that early instars are more susceptible to insecticides than adults which has

previously been seen for *H. halys* (Bergmann and Raupp 2014). Conversely, when tested on eggs, only the azadirachtin + pyrethrins insecticide treatment resulted in significantly higher mortality than the control (Table 3.2). While there was statistical significance, the reduction in hatch rate was not dramatic, from 96% hatching in the control to 91% in the azadirachtin + pyrethrins treatment. The effect of spinosad, neem oil, and permethrins on hatch rate was previously tested for *H. halys* but, compared to our study, lower levels of hatching were observed (Bergmann and Raupp 2014). Differences in methods between studies, such as the age of eggs that were exposed, could explain differences in hatch results. For example, Mathews and Barry (2014) found younger *H. halys* eggs (i.e., 1 d after oviposition) were impacted more greatly by compost tea (i.e., "biologically-active organic matter") than older eggs (i.e., 2-3 d after oviposition) when measuring hatch rate. We exposed egg masses approximately four days after oviposition.

Previous investigation of sublethal insecticidal effects on *H. halys* is limited. Changes in adult mobility in the laboratory after sublethal exposure to insecticides have been investigated (Lee et al. 2013c, 2014b, Morrison et al. 2017b). Other studies assessed *H. halys* injury on insecticide-treated crops, showing that insecticides could variably affect subsequent injury, but they did not investigate whether this was due to direct mortality or sublethal effects (Funayama 2002, 2012, Leskey et al. 2012b, Aigner et al. 2015). Leskey et al. (2014) noted a reduction in feeding by living *H. halys* on certain insecticide-treated apples and peaches, suggesting complete mortality may not be necessary to reduce crop injury. However, without providing insecticide-free food to the insects the authors could not determine the causal mechanism for reduced feeding (Leskey et al. 2014).

By quantifying feeding of surviving individuals on insecticide-free food, we could conclude insecticide repellency was not the mechanism causing feeding reduction. We observed significant reductions in feeding by adults that survived sulfoxaflor exposure (Fig. 3.2). Sulfoxaflor prevented nearly all living individuals from feeding; only two males fed, for a total of only three salivary flanges between them across two weeks (Fig. 3.2). While observing individuals after insecticide treatment, we noticed sulfoxaflor-treated individuals appeared to be trying to feed. Their maxillary and mandibular feeding

stylets however, were splayed out and did not penetrate the food source (Fig. 3.4). No adults exposed to bifenthrin produced any salivary flanges, but this was due to the high mortality by 1 DAT so sublethal effects could not be measured (Fig. 3.2). The other insecticides (pyrethrins, azadirachtin + pyrethrins, and both formulations of spinosad) tested on adults did not significantly change feeding quantity of surviving individuals compared to the control (Fig. 3.2).

We found azadirachtin + pyrethrins and sulfoxaflor reduced molting of 1st instars to 2nd instars (Table 3.2). However, we cannot conclude if these insecticides completely prevented molting because we did not monitor individuals until death or molt but stopped after 5 days. These results indicate, at the very least, that these insecticides delay molting to the next instar. In previous studies, exposure to azadirachtin (Schmutterer 1990, Wang et al. 2014) and sulfoxaflor (Xu et al. 2016) delayed development or inhibited molting of some insect species.

In our study, the effect of an insecticide was not solely characterized by direct mortality or the degree to which feeding was reduced, but a combination of these two factors. To summarize both direct mortality and quantity of feeding, we measured the number of feeding sites/individual, accounting for both dead and living individuals (Fig. 3.3). All insecticides except pyrethrins showed a significant reduction of feeding sites across the first and second weeks after insecticide exposure based on this measure. This means that, due to either death or sublethal feeding effects, exposure to azadirachtin + pyrethrins, organic-certified spinosad, conventional spinosad, sulfoxaflor, and bifenthrin reduced injury/insect compared to no insecticide treatment. When plotted against direct mortality, we get a sense of the level to which direct mortality alone does not capture changes in the amount of injury/insect; this was particularly true for sulfoxaflor (Fig. 3.3). Sublethal effects (e.g., developmental time, population growth rate, fertility, fecundity) of sulfoxaflor have been studied in other insect species (Garzón et al. 2015, Tang et al. 2015, Chen et al. 2016, Colares et al. 2016, Xu et al. 2016, Fernández et al. 2017, Pan et al. 2017), but our study is the first to report sublethal effects on feeding.

Cannibalism of eggs is known to occur in *H. halys* colonies (Iverson et al. 2016) and could potentially confound feeding on seeds if females laid and fed upon eggs while

in their post-treatment containers. However, only seven out of 105 total females laid eggs during the course of the trial and eggs were removed within 24 h after being laid. Therefore, any potential effect of egg feeding on the amount of seed feeding was likely minimal, but we did not quantify feeding sites on eggs.

In the future, it will be important to further investigate how the insecticides evaluated here perform in the field, measuring both direct mortality and sublethal effects. The duration of exposure in our laboratory experiments was longer than what would be expected in a field-setting, particularly for highly mobile adults. Additionally, some of these insecticides rapidly degrade in field settings (Schmutterer 1988, Katsuda 1999, Williams et al. 2003, Spurlock and Lee 2008), and when mortality of adult *H. halys* was compared between the laboratory and field settings it was reduced by >35% (Leskey et al. 2014). Furthermore, caution should be exercised when relying on sublethal effects to achieve efficacy. Despite sublethal effects that may prevent an economic threshold from being reached, insects may still be able to reproduce after insecticide exposure, which in turn could increase the risk of resistance evolution. Hormesis, a phenomenon whereby low levels of exposure to toxic substances can improve pest fitness, could further complicate assessments of efficacy. Studies have found that sublethal doses of sulfoxaflor on green peach aphid, *Myzus persicae* (Sulzer) (Tang et al. 2015) and the small brown planthopper *Laodelphax striatellus* (Fallén) (Xu et al. 2016) produced hormetic effects on reproductive measures. Moreover, we looked at insecticides that are purported to cause less harm to natural enemies. In some cases, only direct mortality was used as the indicator of harm, but insecticides can result in important sublethal effects on natural enemies as well (Delpuech et al. 1998, Desneux et al. 2007, Biondi et al. 2012). Moving forward it will be important to continue studying lethal and sublethal effects of insecticides on the natural enemies in systems with *H. halys*.

Direct mortality alone (Fig. 3.1) or sublethal feeding alone (Fig. 3.2) did not capture the full effect of an insecticide on *H. halys* (Fig. 3.4). It was only by combining these two measures that we obtained a more comprehensive characterization of the crop-protective potential of these insecticides (Fig. 3.3). Perceived efficacy of an insecticide based on mortality alone may therefore be problematic. Many field-based insecticide

trials already use crop injury as the primary means to compare insecticides; however, laboratory studies generally do not measure changes in feeding and are unable to measure crop injury. We suggest that in addition to direct mortality, sublethal effects of insecticides on *H. halys*, specifically those which affect feeding, should be considered in future insecticide efficacy trials, and when developing IPM recommendations and economic thresholds.

Currently, the most widely-used tools for management of *H. halys* are broad-spectrum insecticides (Rice et al. 2014). The increased use of these chemicals to manage *H. halys* can adversely affect natural enemy populations, as evidenced by secondary pest outbreaks and disruptions to IPM programs (Leskey et al. 2012a, Rice et al. 2014). We found that several insecticides that pose less risk to natural enemies can significantly reduce *H. halys* feeding injury, despite lower rates of direct mortality, compared to bifenthrin, a highly lethal insecticide (Fig. 3.3). Having a more accurate estimate of injury/individual after insecticide exposure may allow for less disruptive insecticides (i.e., those causing less mortality to natural enemies) to be considered in management programs, which would be useful in reducing secondary pest outbreaks and for developing more sustainable IPM programs

Tables and figures

Table 3.1 Insecticides and application rates used in bioassays of *H. halys*. All information pertains to labels for use of the product in the United States of America.

Active ingredient	Trade name and manufacturer	Application rate	Insects and crops labeled for the tested rate
Azadirachtin + Pyrethrins	Azera ®, MGK, Minneapolis, MN	4.09 L product/ha (56 oz. product/ac)	High populations of adult insects, such as *H. halys*, on a wide variety of row and orchard crops
Pyrethrins	PyGanic ® EC 1.4II, MGK, Minneapolis, MN	4.69 L product/ha (64 oz. product/ac)	Highest labeled rate for pentatomids in row and orchard crops
Spinosad (Organic-certified)	Entrust ® SC, Dow AgroSciences LLC, Indianapolis, IN	0.73 L product/ha (10 oz. product/ac)	Highest labeled rate in citrus and pome fruits for lepidopteran pests and thrips (Thysanoptera); product is not labeled for pentatomids
Spinosad	Blackhawk ®, Dow AgroSciences LLC, Indianapolis, IN	231.18 g product/ha (3.3 oz. product/ac)	Highest labeled rate in corn and legume vegetables for lepidopteran pests; product is not labeled for pentatomids
Sulfoxaflor	Transform ® WG, Dow AgroSciences LLC, Indianapolis, IN	192.65 g product/ha (2.75 oz. product/ac)	Highest labeled rate in root and tuber vegetables for hemipteran pests (e.g., Cicadellidae and Aleyrodidae)
Bifenthrin	Brigade ® 2EC, FMC Corporation, Philadelphia, PA	0.47 L product/ha (6.4 oz. product/ac)	Pentatomids in a variety of crops, including field and sweet corn, cucurbits and grapes

Table 3.2. Observed proportion of unhatched *H. halys* eggs, mortality of nymphs, and surviving nymphs that molted to 2nd instar by 5 days after hatch (DAH) following topical insecticide exposure to eggs.

	Proportion ± SEM		
Insecticide	Unhatched eggs (n)	Nymph mortality at 5 DAH (n)	Nymphs molted to 2nd instar by 5 DAH (n)
Control	0.04 ± 0.01 (284) ab	0.15 ± 0.02 (274) a	0.83 ± 0.02 (234) a
Azadirachtin + Pyrethrins	0.09 ± 0.02 (277) c	0.67 ± 0.03 (252) b	0.13 ± 0.04 (83) b
Spinosad (Organic-certified)	0.03 ± 0.01 (297) ab	0.96 ± 0.01 (288) d	0.08 ± 0.08 (12) ab
Sulfoxaflor	0.00 ± 0.00 (317) a	0.83 ± 0.02 (317) c	0.02 ± 0.02 (54) b
Bifenthrin	0.06 ± 0.01 (302) bc	0.99 ± 0.01 (283) d	0.00 ± 0.00 (4) NA

Raw means and standard errors are presented, different letters within a column indicate significant differences of least squares means ($\alpha = 0.05$).
Hatch was determined by an empty egg, and nymphal mortality was determined by an inability to walk, eggs and nymphs were held at 25°C 16L:12D.
Each proportion represents the mean across four blocks.
Numbers in parentheses indicate sample size (number of viable eggs, hatched individuals, and surviving individuals) for a given measure.

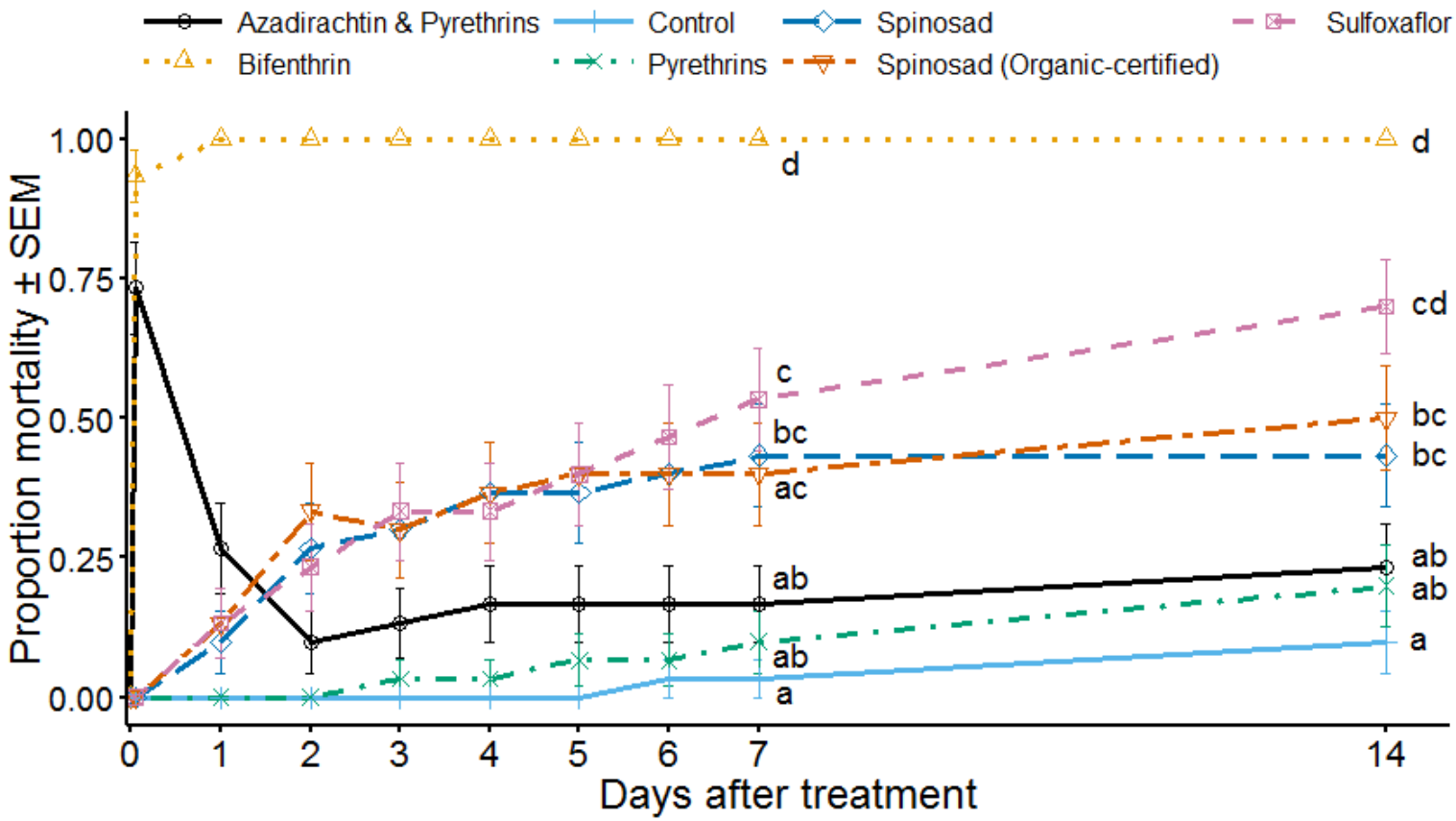

Figure 3.1. Proportion mortality of *H. halys* adults after being exposed residually to insecticides for 24 h. Each symbol represents the raw mean across five blocks. Different letters within a day indicate significant differences of least squares means ($\alpha = 0.05$). Mortality was determined by an inability to walk.

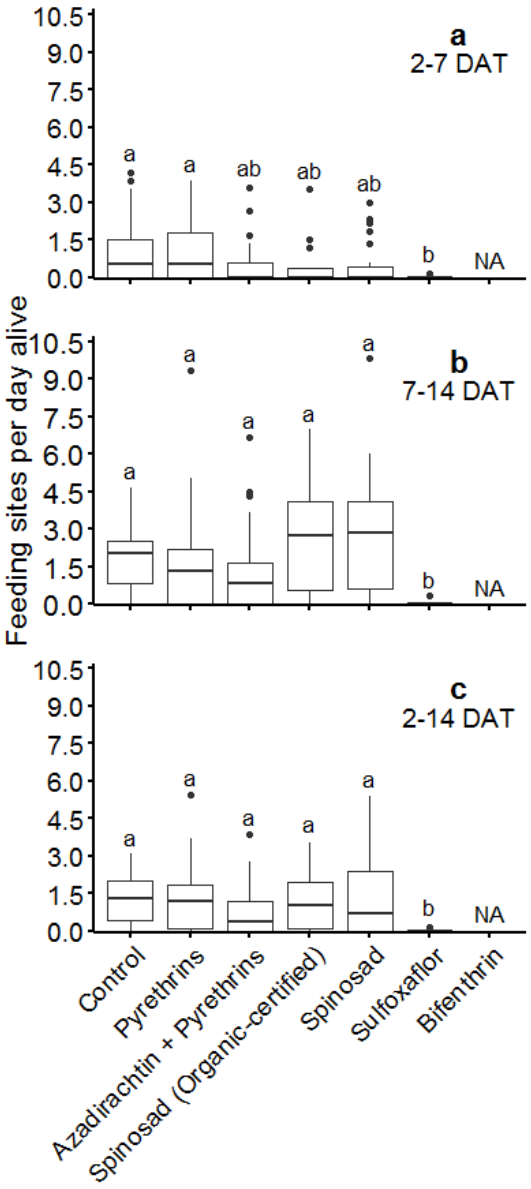

Figure 3.2. Number of feeding sites/day alive for adult *H. halys* at **a)** 2-7; **b)** 7-14; **c)** 2-14 days after 24-h exposure to insecticide residues. Each box represents the raw median and 1st and 3rd quartiles, whiskers extend 1.5 × the interquartile range from the median, and points indicate outliers. Different letters within a graph indicate significant differences of the modeled medians based on a non-parametric Dunn's test with Holm's multiple comparisons correction ($\alpha = 0.05$).

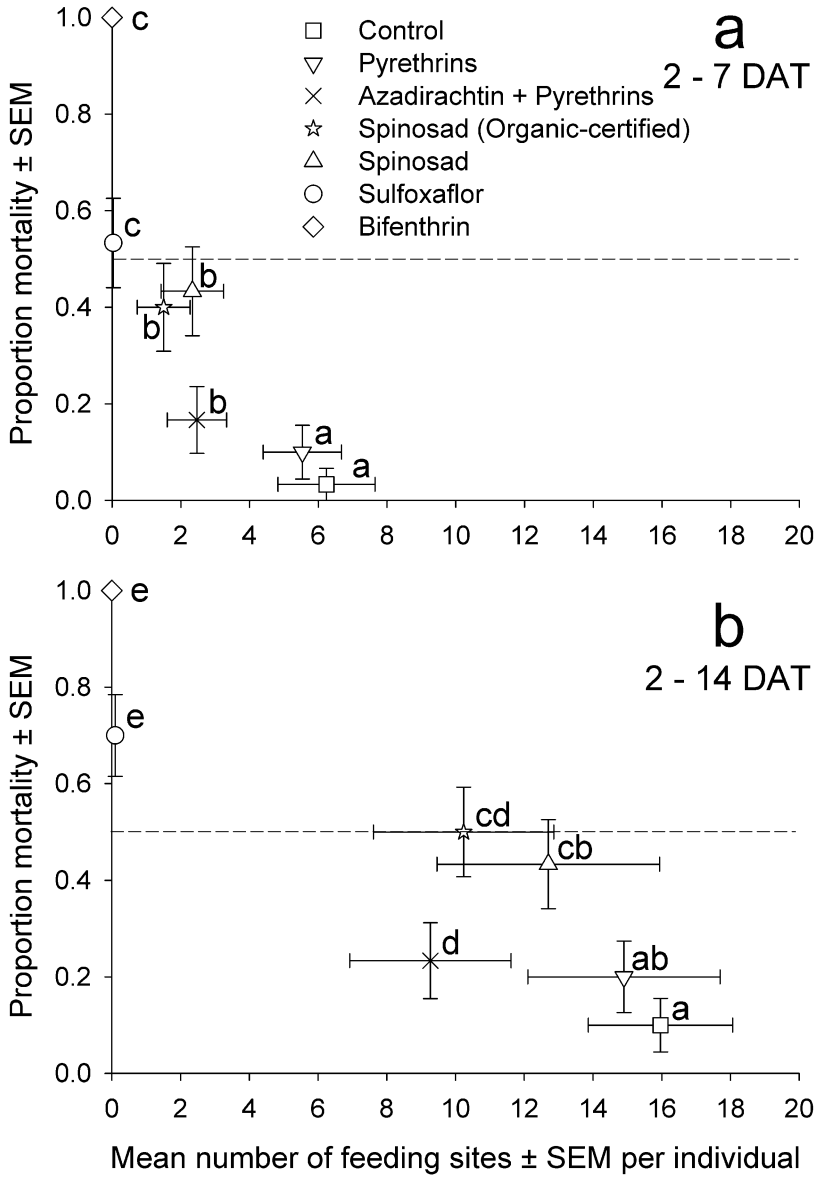

Figure 3.3. Proportion mortality and number of feeding sites/individual for adult *H. halys* **a)** 7 days after treatment (DAT) and 2-7 DAT, respectively and, **b)** 14 DAT and 2-14 DAT, respectively. Each symbol represents the raw mean across five blocks. Different letters within a graph indicate significant differences of least squares means of feeding sites/individual ($\alpha = 0.05$). Dashed lines are plotted at 50% mortality. Mortality was determined by an inability to walk.

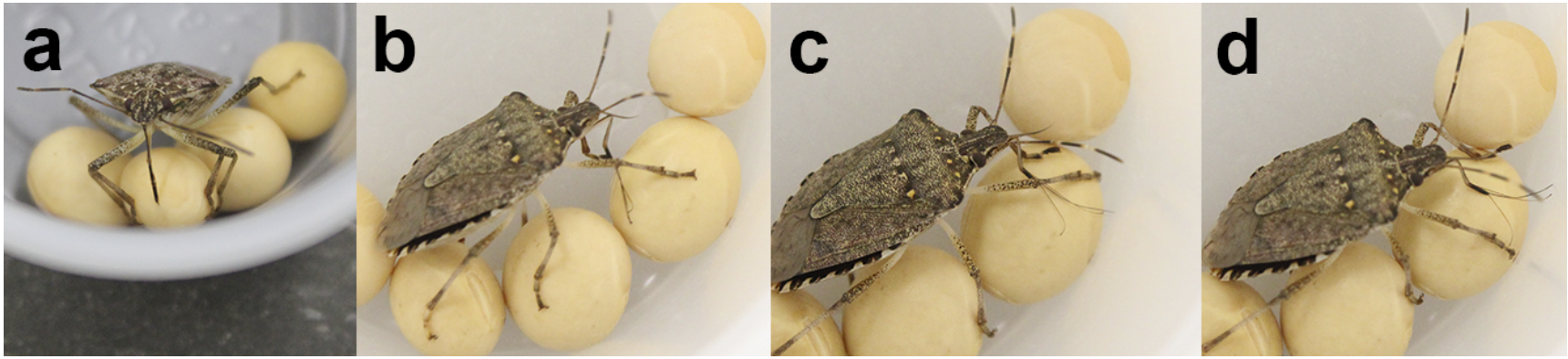

Figure 3.4. Feeding behavior of adult *H. halys* in **a)** an untreated control, and **b-d)** after 24 h residual exposure to sulfoxaflor.

Chapter 4: Implications of linguistic uncertainty in entomology: Insecticide categorization as an instructive case

Introduction

This paper is a call to entomologists to consider the uncertainty that is introduced into our work through the language we use. As scientists, we strive to come to a more certain (i.e., accurate and precise) understanding of the world. As Karl Popper (2012) asserts, we search for objective truth, while understanding complete certainty is unattainable. Developing science together, thus requires transparency about what is and is not certain (Einstein 2007), and the certainty of a piece of information contributes to the determination of its quality (Lesot et al. 2017). The process of building mutual understanding relies on a common language between writer and reader, speaker and listener, to discuss and compare results (Gordin 2015). Language, however, is mutable, uncertain, and an imperfect way of representing the world. Because language is both integral to science and inherently flawed, it is imperative that we strive to recognize and address linguistic uncertainty in our work. For the purposes of this paper, we define *uncertainty* as a lack of accuracy and/or precision in perceptions or communication.

Linguistic uncertainty is one of two major categories of uncertainty identified by Regan et al. (2008) in their taxonomy of uncertainties in the context of ecology. We elaborate on their framework to explore the phenomenon of linguistic uncertainty within entomology. The aim of this chapter is to facilitate recognition, and encourage discussion and problem-solving among entomologists regarding linguistic uncertainty, as it plays an important role in communicating and conducting science. We illustrate our assertion that linguistic uncertainty is commonplace and consequential through an analysis of how insecticides have been described and categorized in the top entomological journals between 2006 and 2016.

We focus on written language because it is the channel entomologists most commonly use to share and distribute knowledge amongst ourselves. Furthermore, it provides a searchable body of communication for the empirical analysis in this paper.

First, we briefly introduce key ideas about communication, language, and uncertainty. Second, we highlight recent literature exploring linguistic uncertainty in natural sciences. Third, we explore the drivers and effects of linguistic uncertainty in scientific writing, broadly. Seeing and understanding general causal patterns may help writers in the future to identify and recognize specific causes of linguistic uncertainty in their own work. At times, linguistic uncertainty is desirable, and complete linguistic certainty is an unattainable goal. Therefore, we also discuss potential positive and negative consequences of linguistic uncertainty. In the final section of the paper, we use an illustrative case study − the categorization of insecticides, specifically the descriptor *broad-spectrum* − to demonstrate and discuss the presence and consequences of linguistic uncertainty in entomological literature. We report that: the system, or lack thereof, for classifying insecticides is rife with linguistic uncertainty; broad-spectrum embodies many types of linguistic uncertainty leading to inconsistent categorization of insecticide active ingredients and insecticide subgroups; and this linguistic uncertainty has allowed a great deal of unacknowledged subjectivity to pervade in scientific papers. We conclude with suggestions for how to address linguistic uncertainty broadly and within the field of insecticidal categorization.

Communication and uncertainty

Communication

In order to compare individual experiences of the world, we must share our perceptions with one another. Language is a "system for describing perceptions" (Zadeh 2004). Language allows for rapid sharing of accumulated knowledge but not without some degree of distortion. The first layer of distortion occurs because abstract forms, such as words and numbers, can only imperfectly convey our experiences and the world. Translation between two logic systems, such as from perceptions to language, reduces the accuracy and precision of the conveyed perception. The second layer of distortion arises from the dynamic and individualistic nature of language.

Language is in a constant state of change. The number of words in the English lexicon has increased 70% from 1950 to 2000 (Michel et al. 2011). In 2000 there were

approximately 1,022,000 words in the English lexicon, nearly double the 544,000 words in 1900 (Michel et al. 2011). Not only do new words arise and unused words fall away, but one word may have many meanings (polysemy) and the meanings of words change over time (semantic change). Britton (1978) analyzed the 100 most frequently used English words for polysemy and found 32% of them were "lexically ambiguous," i.e., they had more than one meaning. Within that set of ambiguous words, the number of meanings a word had ranged from 1 - 31. Blank (1999) outlines six drivers of semantic change: new concepts, abstraction, sociocultural change, close conceptual relations, reduction of lexical complexity, and emotionally marked terms. Hamilton et al. (2016) found two factors that predict semantic change. Words that had higher levels of polysemy had higher rates of semantic change, and words that are used more frequently had lower rates of semantic change. On top of these dynamics more than half (52%) of the English lexicon was found to be completely undocumented in standard references, so-called "dark-matter," (Michel et al. 2011). How, then, does this highly dynamic, subjective, undocumented, and ultimately uncertain language effect entomological communication? Do ambiguous and indeterminate terms provide flexibility or increase confusion?

Uncertainty

As individuals, our ability to sense and process the attributes of the world is bounded by our sensory organs and mental abilities and capacity (Zadeh 2004). These bounds constrain the certainty of our knowledge of the world. If we assume that there exists a material world, independent of individual constructions and perceptions of the world (i.e., a realist philosophical framework, as opposed to an idealist), we assume there is a reality which can be empirically discovered. Certainty about this world arises through the convergence and agreement of perceptions among senses and people, and across time (Eckhardt 1981). The scientific process is employed to compare perceptions and create knowledge about the world (Einstein 2007). The empirical nature of the scientific process is, in part, what makes scientific knowledge useful. If a phenomenon is repeatedly observed, and individual measurements converge to some degree on a specific point, we can assert more certainty about the true nature of the phenomenon. We will never know

the world with complete certainty (Popper 2012), so scientific knowledge often comes paired with a level of uncertainty (i.e., error) associated with its measurements.

As mentioned, Regan et al. (2008) previously outlined a taxonomy of uncertainties discussed in the context of ecology. They divide uncertainty into two broad categories, *epistemic* and *linguistic uncertainty*. We use their framework and terminology throughout the rest of the paper, and thus begin with a summary of their taxonomy, supplemented with our own entomologically-relevant examples.

Epistemic Uncertainty

Epistemic uncertainty occurs when knowledge about a system is not complete (Regan et al. 2008). This form of uncertainty is more familiar to most scientists, so we describe its six major forms only briefly. *Measurement error* represents the uncertainty in the variability of individuals in a population and is reported through mechanisms such as confidence intervals around point estimates. *Systematic error*, or bias in experimental equipment and procedures, is addressed via rigorous examination of experimental procedures, comparison across independent studies, and replication (for more, see also Hurlbert (1984)). When a system is irreducible to a deterministic one (e.g., Heisenberg's uncertainty principle), *inherent randomness* introduces uncertainty, however truly random systems are rare in ecological and biological systems. To address uncertainty due to changes in a measured system through time, space, or other variables (i.e., *natural variation*), work is repeated across temporal, spatial, cultural or other scales to understand and measure variability across those scales. Validation studies, such as ground-truthing, seek to reduce *model uncertainty*. When the judgment of an expert is used in place of empirical data, such as when data are error prone or scarce, *subjective judgment* arises.

Linguistic uncertainty

Linguistic uncertainty is less commonly recognized, quantified, and discussed by entomologists, yet merits great attention because language is the medium by which we share and advance our science. At first glance, it may seem that to write clearly one just needs to follow prescriptive rules about grammar, mechanics, and structure (e.g., Day and Gastel 2011). A well-structured, grammatically correct paper, can still fail to

communicate correctly, or certainly however. Just as knowledge carries uncertainties, communication about that knowledge does as well. Recognizing that language is an imperfect way of representing reality, Regan et al. (2008) define *linguistic uncertainty* as the uncertainty that arises in language and divide it into five types. The complex and dynamic nature of language may make complete certainty in communication impossible, however, as with epistemic uncertainty, there are means to acknowledge and reduce linguistic uncertainty. When a word has more than one potential meaning, it can be explicitly defined to reduce *ambiguity* for a given context. The term "significant" would be considered ambiguous if its intended meaning (e.g., statistically significant, or subjectively perceived to be important) is not clarified. *Context dependency* increases uncertainty when the context a statement should be understood in is not stated. For example, the sentence, "Syrphid flies are predators" lacks context because it does not specify which life stage (larva) is predatory. *Underspecificity* increases uncertainty when the necessary degree of specificity to accurately interpret a statement is not included. To say "the specimen was found east of Minneapolis" is underspecific because it would not be possible to determine exactly where the specimen was found.

Indeterminacy of theoretical terms arises because language changes over time, also known as semantic change. For instance, in the 16th century the term *pest* arose from the term *pestilence*, or a fatal epidemic, specifically the bubonic plague, and referenced the plague or a contagious disease. Through semantic change, *pest* has since come to mean noxious people or things (Onions et al. 1966). Mediating the potential changes a word may go through over time is not easily done. *Vagueness* is determined by the nature of the words or predicates used to define a term. If a definition allows for borderline cases, the term is vague. A classic example of vagueness, due to vague predicates, is illustrated by the sorites paradox, or paradox of the heap. The paradox begins by asserting two premises as true, that: 1) 1,000 grains of sand is a heap, and 2) a heap minus one grain of sand is still a heap. If the assertions are repeated, removing one grain of sand at a time until a single grain remains, the paradox arises: one grain of sand is a heap. Thus the word *heap* is vague; other common vague words include tall, old, large, and color terms such as red. To resolve this, the impulse may be to define a sharp boundary, say, only

piles of sand with 800 grains or more are heaps. However, such a bivalent logic may not always be satisfactory. Does the one grain of sand difference from 799 to 800 grains *really* surpass some meaningful limit? This paradox illustrates the difficulty of defining and dealing with vague words. Fuzzy logic and supervaluations have been employed to deal with linguistic uncertainty from vague words.

Lessons learned from linguistic uncertainty in the natural sciences

Linguistic uncertainty has not been explored in entomology, but research in other natural science fields implies that we need to consider it within entomology as well. Here, we present illustrations from prior studies that demonstrate linguistic uncertainty is both prevalent and consequential. Not all studies used a framework of uncertainty to describe or discuss their work. So, for clarity and consistency we apply the terms Regan et al. (2008) propose to these studies and to our analysis of entomological literature.

Regan et al. (2000) argue that classification of endangered species according to vague terms presents problems. It is not the inherent vagueness of the categories that is problematic, but rather the sharp boundaries that arise between categories in ways that do not make biological or managerial sense. For example, 80% population decline of a species in 10 years was the boundary between the categories of *critically endangered* and *endangered*. Regan et al. (2000) state strictly differentiating between populations with 79% and 80% decline in 10 years is not useful, nor does it make biological sense. Additionally, they argue that categorization is predicated on accurate and precise data, which can be difficult to obtain. This can lead to misclassification or an inability to classify species at all, especially those which fall on or near sharp boundaries between two categories. To address the problem of categorizing species when they fall on or near boundaries Regan et al. (2000) designed a fuzzy set-theoretic alternative with membership functions that reduce the sharp boundaries between categories. With this, one can determine when a species is certainly critically endangered, or certainly not critically endangered and, importantly, provides greater information about species that fall in between these two clear categories.

Herrando-Pérez et al. (2014) state that the lack of a convention of nomenclature for ecological terminology "handicaps the progress of ecology." Their main concerns are that linguistically uncertain terms prevent synthesis across studies, impede the exchange of ideas, and contribute to redundant research. To address ambiguous terms, redundant terms, and the general lack of a standard lexicon, they propose a convention of ecological nomenclature. They recommend an advisory committee with oversight be created, and a method by which terminology can be proposed, assessed, and searched, be established.

Fraser et al. (2015) found, through a study on the classification of woodland birds, that ecological terminology is not consistent and inconsistences influence the results of studies. The authors determined that the category "woodland bird" is not understood uniformly, being both ambiguous and vague. A key reason for inconsistences in classifying birds was due to inconsistencies classifying woodland vegetation. Also contributing were the variety of mechanisms survey respondents used to determine bird category membership. Bird occurrence in woodlands, bird traits (e.g., nesting or foraging in woodlands), classifications found in field guides or journal articles, and exclusionary criteria (e.g., not an introduced species) were criteria used to categorize birds. Observed consequences of linguistic uncertainty were an inability to compare results across studies, and the potential to change management decision-making depending on which definition of woodland bird was used. Interestingly, when the authors surveyed woodland bird experts, all 69 respondents acknowledged the term is used inconsistently; 80% of respondents did not find this problematic, and 57% of respondents believed a unified list of woodland birds would be problematic and prohibitive to certain research questions.

Carey and Burgman (2008) present an interesting case that shows how levels of agreement between risk assessors increase as linguistic uncertainty decreases. While facilitating a risk assessment workshop about hazards to a marine protected area, the authors measured the level of agreement before and after linguistically uncertain terms were discussed and clarified. They found vague terms like *few* and *many*, and ambiguous terms *hazard* and *marine debris* arose. Additionally, the context (i.e., specific locations within the intertidal region) was not always appropriately specified and a lack of data on the level of recreational boating resulted in a problem of underspecificity. Their study

found that some disagreements were occurring simply because of poor communication, and greater agreement could be achieved through the reduction of linguistic uncertainties. For example, by clarifying meaning, collaboratively delineating categories, specifying contexts, and defining terms.

Crump et al. (1999) identified confusing terminology related to "biological weed control". They sought to standardize and clarify ambiguous, illogical, and contradictory terms by proposing a hierarchical classification of existing terms and introduction of a few new terms. They point out that the baggage certain words, such as chemical and synthetic, carry could affect the public trust, and ultimately the fate of one type of control over another. They conclude that allowing terms to be defined individually in each paper, rather than insisting on a common definition, complicates communication and increases confusion and "[c]lear, meaningful terminology is one essential step in gaining public confidence" (Crump et al. 1999, p 94)

Ostry et al. (2011) discuss a forest tree disease category, "decline". They state the term is ambiguous, decreases clarity in communication, and has no practical value in a management context. They recommend discontinuation of the use of "decline" as a disease category, systematically studying the complex causes of tree death, and subsequently creating more certain categories of tree diseases based on research findings about the major determinant of tree death.

Horowitz et al. (2009) introduce their book on biorational control of pests by admitting that the term "biorational" has been used inconsistently through history, and is imprecise. They detail the general confusion about the word and note that no standard definition of biorational exists. The authors offer their own suggestion of a definition but, interestingly, believe that it will be difficult to stabilize a definition of biorational without the United States Environmental Protection Agency (EPA) proposing a definition.

Eilenberg et al. (2001) found different disciplines researching biological control use different terms which has led to confusion among scientists and other non-specialists such as extension agents and regulatory officials. They proposed a set of terms and definitions which they hope will improve the exchange of information between disciplines.

Sources of linguistic uncertainty

This paper does not attempt to determine *why* linguistic uncertainty occurs in *specific* cases, but rather identifies common causes of linguistic uncertainty. Language is inherently inaccurate and imprecise and can only imperfectly convey meaning between separate entities. Across national, language, regulatory, and cultural boundaries the definition of a word may change. Specifically in scientific writing, we find many other possible reasons for linguistic uncertainty to occur. Many journals have word limits, and requiring writing to be succinct may come at the expense of sufficient context. A word which can concisely communicate a complex topic may actually perpetuate linguistic and epistemic uncertainty due to oversimplification. An author can avoid, ignore, or oversimplify epistemic uncertainties by using linguistically uncertain words, in essence, increasing the appearance of certainty when epistemic precision and/or accuracy may be lacking. Linguistically uncertain statements could be utilized to make scientific work more generalizable across different contexts, yet leaving out important clarifications or assumptions may lead to unsound extrapolations. Conversely, highly technical and certain language may inhibit understanding of scientific work by other stakeholder groups. In an effort to make the science more accessible or applicable to decision-making contexts it may be necessary to translate scientific results into less certain terms. Yet, when common words are used in uncommon ways non-experts may unknowingly misunderstand the meaning of a statement (Fischhoff and Davis 2014).

Writers may be ignorant of the uncertain language they use for any number of reasons (e.g., level of scientific expertise, native language). There are no standard measurements of error that can be paired with language to report the precision and accuracy of words. This means the level of linguistic uncertainty a term embodies is determined subjectively based on a writer or reader's background knowledge and expertise. In other cases, writers may deliberately employ linguistic uncertainty. Using non-falsifiable, vague words can keep an idea alive when personal beliefs or world-views, or investments (time, professional, money) are threatened. Linguistic uncertainty can also be used to create obstacles that inhibit, prevent, or discredit research (e.g., the

politics around the terms *global warming* and *climate change* in the United States). There is often no way for writers and readers to double check that accurate and precise communication has taken place between them. Oblivious to miscommunication, uncertainties can then compound. The number and diversity of readers of a scientific paper can also increase the paper's linguistic uncertainty.

Consequences of linguistic uncertainty

Uncertainty inevitably will arise in our communication. The impacts of linguistic uncertainty on communication and science vary depending on the specific type of uncertainty and the circumstance. They can have desirable or undesirable effects. Here, we have compiled a non-exhaustive list of possible consequences of linguistic uncertainty in entomological writing.

Linguistically uncertain words in a paper increase readers' power to define and interpret the research. Absent a definition, readers will apply their own choice of definition and understanding of a term, which may or may not match the writers intended meaning. In some cases, this may be desired, such as when flexibility to extrapolate results to myriad contexts is needed. Allowing readers to take liberties interpreting the meaning of research could encourage creative new lines of questioning or discovery of new understandings. However, allowing for variable understandings could allow readers' biases to arise in an unnoticed way. That flexibility can also give readers the ability to misappropriate results in scientifically unsound ways. It is important to consider the level of subjective interpretation words allow. Differing levels of uncertainty may be unacceptable depending on the stakes of the decisions made based on the research.

It is well documented that language can be used to shape the framing and interpretation of knowledge. Commonly, outcomes are compared to a neutral reference point in order to judge the outcome as positive or negative (Tversky and Kahneman 1981). Tversky and Kahneman (1981) experimentally showed that changing the reference point can alter the perception of gain or loss. Thus, the reference points (i.e., positive and negative controls) that scientists choose, can change the readers' perceptions of risk or benefit. Some types of linguistic uncertainty bring writing closer to persuasive rhetoric

(pathos) rather than descriptive logic (logos). This shift can critically reduce or undermine the validity of the information being communicated (Jamieson 2017). Additionally, pathos impedes the process of science by reducing reproducibility and rigorous interpretation and discussion of scientific work. While we do not espouse science as a wholly objective discipline, when linguistic uncertainty is used deliberately to conceal biases in scientific writing, it threatens the credibility of the science.

Linguistically uncertain terms, when recognized as such, can be used to highlight areas where more empirical work is needed, where expert disagreement exists, or where other types of inquiry (e.g., values-based analysis) are more appropriate. If a reader can recognize and see through this veil of uncertainty, it will assist them in weighing the strength of the results and in discovering where epistemic uncertainty exists. Seeing where linguistic uncertainty is causing uncertain communication is important because linguistic uncertainty can contribute to compounding uncertainties. Like a game of telephone, readers misunderstand writers, inappropriately cite their work and make flawed assumptions based on the work, become writers themselves, and the process repeats.

Some argue strongly for reductions in linguistic uncertainty (e.g., Peters 1991, Herrando-Pérez et al. 2014), believing the quality and efficiency of scientific discourse decreases with greater linguistic uncertainty. While others believe that lexical richness is good and prescriptively standardizing terminology is a bad (Hodges 2008). Hodges (2008) state that precise definitions are rarely necessary and can hinder scientific advances by preventing certain lines of questioning, which ornithologists seem to agree with (Fraser et al. 2015). Even when well defined terminology exists, and writers are aware of the terms, they may not use them (Kritsky 2001). We believe that linguistic uncertainty cannot be qualified solely as good or solely as bad. Linguistic uncertainty can have positive *and* negative consequences, and complete agreement as to which consequences are good or bad does not exist. Drawing such a value-based line lies outside the realm of scientific questioning, but is not inconsequential to science.

Classification: Categories and dichotomies

When communicating or thinking about the world we use shortcuts. Categories are shortcuts, they convey meaning without extensive description. Our concept of a specific category, such as *insect*, is not independent from our acquaintance with members of that category. While the dictionary definition of *insect* indicates the animal has six legs, familiarity with insects and the world allows us to still call an ant who has lost two legs an *insect.* This individually unique and vast prior knowledge builds the matrix of our mental representation of a typical member in a category, and guides where we believe the delimiting bounds of the category should be (Taylor 1995). Individual representations of a category are never identical, yet in general, the overlap of individually created meanings for a category is substantial enough to communicate at an adequate level of precision and accuracy.

Dichotomies present the smallest set of categories, just two. The simplicity of an either/or choice provided by a dichotomy can be attractive and convenient and can offer a strong argument for or against something. Would you prefer something that is safe or unsafe? However, the bivalent logic of dichotomies can be miss-assigned, ignorantly or persuasively. Presenting only two options can hide the continuum and inherent vagueness a system embodies.

Taylor (1995) describe three ways people regard categories. They include, nominalism which asserts categories do not reflect reality, but only linguistic convention. That is, the only thing in common between *beetles* is their name. Realism, which asserts categories reflect reality, and exist independent of language. For example, *beetle* arose from a pre-existing category. Lastly, conceptualism which asserts categories arise from mental concepts which are based on reality and expressed through language, a merging of nominalism and realism. We take a more conceptualistic stance that in circumstances of diffuse continua (much of the world), drawing boundaries between categories is subjectively shaped by language and culture (Taylor 1995), and we add shaped also by experience. Categories are bounded by our ability to perceive and think about the elements to be categorized. Linguistic, cultural, and experiential forces can narrow or broaden the number of categories an individual perceives.

Another factor contributing to categorization are the traits of interest or concern for a set of items. For example, insects can be categorized taxonomically, by geographic range, and/or by the level of harm or benefit they confer to humans. Determining which traits are of interest and which type of categorization will be used is a subjective decision. Research results and implications are framed, in part, by the type of categories used, which in turn strongly influences management. Categories are powerful, and erroneous classification of something can impede scientific thought (Townend and Walker 2006). Categories can illuminate the cognitive representations of the world a writer uses to frame their work. They may help or hinder communication, and reduce or increase uncertainty.

Case study: Broad-spectrum insecticides

Insecticides are categorized and described by a variety of mechanisms. These categorization schemes in many ways make sense, yet no scheme can capture the entire profile of an insecticide. In some cases, categories are structured by bodies such as governments, non-governmental certifying agencies, or research communities (Table 4.1). The regulating bodies can outline the category or set of categories and/or certify which insecticides are or are not in a category. Outside of this list of formalized categories, many far less structured categories are commonly used to describe or classify insecticides (e.g., biorational, botanical, soft, persistent), often lacking consensus or guidelines for their use. Across both types of categorization, regulated and unregulated, there is little to no inter-category overlap in the criteria used to determine membership into a category.

Methods for case study

Case and literature selection

To investigate categorization of insecticides, we chose one term to explore in more detail: *broad-spectrum*. We chose this term because of the high prevalence of its use in the entomological literature, the length of time it has been in use (Fig. 4.1C), and its vague and ambiguous nature. We surveyed recent papers and the oldest literature

containing the terms *broad-spectrum* and *insecticide*. To narrow down the recent publications, we choose the top 11 Entomology journals based on the Thomson Reuters Journal Impact Factor in 2015, whose descriptions included some mention of agricultural entomology or pest management (Table 4.2) (*Pesticide Biochemistry and Physiology* was among the top journals, however, due to limited access it was excluded from the pool). Then, using the Web of Science database, to search the title, abstract, and keywords of all the publications in these 10 journals from 2006 to 2016, we identified all articles containing both *broad-spectrum* and *insecticide*. This returned 64 papers, however in four papers broad-spectrum was not used in relation to insecticides so those papers were not included in our analyses.

Textual analysis of the literature

We used text analysis methods that are commonly seen in the social sciences to identify and characterize patterns of language use and meaning in these texts (Feldman et al. 2004, Czarniawska 2011, Feiock et al. 2016). We used these methods to survey the resultant 60 entomological peer-reviewed papers to identify:

1. **Formal definitions** of *broad-spectrum insecticide*. This was accomplished by seeking explicit definitions in one of three ways:
    - To find key antecedents for its current use, we searched the 60 recent articles for citations associated with sentences containing *broad-spectrum*. We reviewed the title of the cited article to determine what portion of the sentence was likely associated with the citation. Often citations related to an insect species rather than an insecticide. If, based on the title, we could not determine what was referenced or thought the citation may have clarifying information about insecticide classification, we retrieved the citation's full text and searched it for any clarifying information about the meaning of broad-spectrum.
    - Web of Science did not return any papers containing *broad-spectrum* and *insecticide* prior to 1981 so we searched for the earliest use of the adjoining term *broad-spectrum insecticide* as an insecticidal category and any explanation or definition of the category, using Google Scholar and

Google Books limiting the search to texts prior to 1960. These two search engines search the entire text of a book or article but do not provide the full text. Google Books returned 16 books and Google Scholar 4 texts. If we were unable to obtain the full text via other means to verify the source and date, or found the reported date of a text was incorrect (which was common when searching Google Books) the source was not included. We obtained 1 of the Google Book results and 2 of the relevant Google Scholar texts and reviewed them for explicit definitions of *broad-spectrum insecticide*.

- We compiled a subset of definitions of *broad-spectrum insecticide* from standard entomological references in print and on the Internet (found from a Google search of *broad-spectrum* insecticide).

2. **Inferred definitions** of *broad-spectrum insecticide*. Where formal definitions were absent we recorded terms that conveyed an inferred meaning to the category of *broad-spectrum insecticide*. These terms were taken from the sentence in which *broad-spectrum* was used, or the sentence on either side. Implicit meaning of broad-spectrum category membership came from associated terms, identified as the nouns that broad-spectrum modified (except for Insecticide Resistance Action Committee (IRAC) insecticide groups, subgroups/chemical classes and active ingredients, or trade names which are analyzed in the membership and non-membership section) and other adjectives and nouns that applied to the noun that broad-spectrum described. Implicit meaning of *non*-broad-spectrum category membership was determined by disassociated terms, identified as nouns and adjectives that broad-spectrum was compared to or directly stated as being in opposition to (except for IRAC insecticide groups, subgroups/chemical classes and active ingredients, or trade names).
3. **Membership and non-membership** in the broad-spectrum category. We inventoried the specific IRAC insecticide groups, subgroups/chemical classes and active ingredients that were described as *broad-spectrum* (members of the

category), or were being compared to or distinguished from *broad-spectrum* (non-members of the category). Comparing the overlap of terms in these two inventories allowed us to see how consistently broad-spectrum category membership has been assigned in the subset of papers we reviewed.

4. **Definitions via contrast and connotation**. Using a well-established qualitative methods analytical technique of thematic coding and categorizing, we created three categories to evaluate how the term *broad-spectrum* was being used. This is a classic technique of inductive analysis and grounded theory development from textual data, and involves first, gathering terms or phrases and grouping them into related ideas, then provisionally naming and defining those groups, and finally iteratively testing group members against group definitions until there is both excellent alignment of the definition to members of the group and the creation of an analytically useful set of categories (Glaser and Strauss 1999, Charmaz 2006). These categories covered the full range of uses with minimal overlap or difficulty differentiating between them. They included 1) descriptive use, 2) comparative use (i.e., in relation to another category), or 3) evaluative use (i.e., associating a negative or positive suite of characteristics to something). In the evaluative use category, we qualitatively assessed positive and negative valences, assuming a human-centric, profit-driven perspective.

Results and discussion of case study

Formal definitions

The use of the term *broad-spectrum* markedly increased around 1950 (Fig. 4.1A). As an adjective, *broad-spectrum* most commonly modifies words pertaining to antibiotics (Fig. 4.1B). We speculate that the *broad-spectrum insecticide* category arose soon after the term *broad-spectrum antibiotic* ("active against a wide variety of organisms" (Martin 2015)) began to be widely used (Fig. 4.1B). However, in the process of adopting the adjective from the field of bacteriology a formal entomology-specific definition was never proposed. We found no seminal paper which coined the term *broad-spectrum*

insecticide. The earliest record in the electronically searchable and verifiable literature describing an insecticide as broad-spectrum (Cooper 1958) was written as if the term was already assumed to be understood in the lexicon:

> "Research work in the pesticide field continues, some directed toward specific chemicals for specific insects, some toward a more broad spectrum insecticide." Page 41

> "When working toward a broad spectrum insecticide the problems of biological balance must be considered, and in the minds of many, the answer to a broad spectrum material will be finally answered through the systemic field. It is every company's hope to achieve an acceptable insecticide, which when used correctly, will give control of all the insects we wish to control, and yet not harm those insects beneficial to mankind." Page 41

From that point forward this term was clearly integrated into the entomological lexicon (Fig. 4.1C), without however a unifying definition in either the oldest literature or in recent papers. None of the 60 recent papers, 3 oldest references, or 14 references cited within them that we also read, provided an explicit definition of what broad-spectrum means when it describes insecticidal activity or as an insecticidal category. In the expanded search for definitions of *broad-spectrum insecticide* in standard entomological references (Torre-Bueno et al. 1989, Capinera 2008, Gordh and Headrick 2011), pest management books (Ware and Whitacre 2004), and extension websites (Cornell University Cooperative Extension 2012, National Pesticide Information Center 2015, University of California Agriculture and Natural Resources Statewide IPM Program 2016) we found at times conflicting, definitions (Table 4.3). In brief, explicit definitions of *broad-spectrum* included insecticides which kill a *wide range* of target organisms, *large number* of unrelated species, *wide range* of pests, *more kinds* of insects than other insecticides, and insecticides that effect pests and beneficials, or that are effective against *all* insects (Table 4.3).

The term *broad-spectrum insecticide* is defined in different, and at times conflicting ways in standard references (Table 4.3). Terms lacking one standard definition are considered "ambiguous" according to Regan et al. (2008)'s taxonomy of uncertainty. The ambiguous nature of broad-spectrum was not acknowledged or addressed in any of the papers we read. Authors did not specify which definition of *broad-spectrum* should be applied to their work. This indicates that writers are assuming a uniform understanding of the word broad-spectrum.

Not only is the term *broad-spectrum insecticide* ambiguous, definitions of the term are vague. Vague terms are defined with words that allow borderline cases (Regan et al. 2008). To illustrate this, consider the definition of *broad-spectrum insecticide* based on its three component words as defined by Mirriam-Webster. Broad: widely applicable or applied (Merriam-Webster.com 2017a), spectrum: a continuous sequence or range (Merriam-Webster.com 2017b), and insecticide: an agent that destroys insects (Merriam-Webster.com 2017c). Combined, one could interpret that a broad-spectrum insecticide is one which "destroys a wide range of insects." This definition is vague mainly due to two words, "destroy" and "wide". To destroy, must the insecticide kill the entire population? A percentage of the population? Can destruction from an insecticide include sublethal effects which reduce fitness? Likewise, where is the cutoff for wide? Destruction to more than just one species? More than 10 families? Or is the width of the range of insects determined by more anthropocentric means like destruction of pests versus beneficials? Within the explicit definitions we found, additional vague terms are used, such as: affect, large number, unrelated, effective, good insects, wide range, killer, several kids of insects (Table 4.3).

Broad-spectrum contains further linguistic uncertainty because it is inherently a relative term: *broad* in relation to what? Relativity is not specifically addressed in Regan et al. (2008)'s taxonomy. However, in this example the relative nature of *broad* increases the likelihood *underspecificity* and *indeterminacy of theoretical terms* occurring. Underspecificity arises when authors do not explicitly state what broad is in relation to, or which insecticides they consider to be or not be broad-spectrum. Additionally, being a relative term makes *broad* unstable across time, increasing the chance of semantic change

and therefore greater linguistic uncertainty. The suite of insecticides available for use, and thus available to be compared to, changes over time. New information may change what constitutes *destruction* and values may change what constitutes *wide*. These fluctuations may not fundamentally change the definition of the term but may cause readers in the future to see the boundaries of the category differently.

Inferred definitions

Where an explicit definition is lacking, terms associated and disassociated with broad-spectrum could assist a reader in building a definition. However, across papers, contextual cues to intuit the meaning of broad-spectrum insecticide were diverse and at times contradictory (Fig. 4.2). Our inventory of terms associated with and distinguished from broad-spectrum provides useful information about the matrix of mental representations of the category, and allows us to compare the delimiting bounds of *broad-spectrum* across papers (Fig. 4.2). The specific nouns and adjectives associated or disassociated with broad-spectrum, present at a single word level, clear, though not always correct, delineations. For example, within a single paper seeing that *older* was associated with broad-spectrum, a reader could assume that all broad-spectrum insecticides are older, or that being old is a condition that determines whether an insecticide is broad-spectrum. However, from a cumulative level across all the associated and disassociated words, an inferred definition of broad-spectrum is harder to build. Terms from each column were not mutually exclusive. For example, certain *organic* and *reduced-risk* insecticides (terms disassociated from broad-spectrum) would be considered broad-spectrum based on terms that were associated with broad-spectrum (e.g., conventional, environmentally persistent, synthetic) (Fig. 4.2). This indicates that writers in entomological journals do not uniformly understand the term broad-spectrum. It would not be possible for someone to build a logically consistent definition of what broad-spectrum means based on what papers infer broad-spectrum to be.

Based on the level of disagreement we found in explicit and implicit definitions of *broad-spectrum insecticide* there is clearly no uniform understanding of the word. Writers and readers are likely unaware of the miscommunication occurring between them. Relying on reader-created definitions in the case of *broad-spectrum insecticide*

decreases certainty in communication because readers' contexts (e.g., cultural, linguistic, historical) differ from the writer's and intuited definitions are highly reliant on readers' understanding other undefined insecticide terms as well. This linguistic uncertainty becomes particularly problematic when the rational, premise, conclusions, and/or implications of a paper rely heavily on the broad-spectrum insecticide category.

Membership and non-membership

Significantly, several nouns (shown in the middle column of Table 4.4) were described both as broad-spectrum and as a category being distinguished from broad spectrum. Further investigation revealed two reasons for this discrepancy.

1) *Broad-spectrum* largely was used to modify the term *insecticide* or a synonymous term (e.g., a specific insecticidal subgroup/chemical class or active ingredient). In fewer instances, *broad-spectrum* was used to modify *activity* in relation to a specific insecticide, subgroup/chemical class, or active ingredient. We found that the relationship between the *adjective* broad-spectrum activity and the *category* broad-spectrum insecticide was not clear (Table 4.5). It seems counterintuitive that an insecticide described as having a broad-spectrum of activity is not considered a broad-spectrum insecticide. However, insecticides with broad-spectrum activity only in a narrow taxonomic group seem to be able to exist in this space. Yet the question remains, when does something with broad-spectrum activity become a broad-spectrum insecticide?
2) In other cases, such as with the IRAC insecticide subgroup *neonicotinoids* and some active ingredients in this subgroup, the discrepancy was reflecting the ability to subjectively determine the boundaries of the broad-spectrum insecticide category (Table 4.6). Typically, three to four IRAC subgroups, including neonicotinoids, were listed when an author gave examples of broad-spectrum insecticides (Sisterson et al. 2008, Hamby et al. 2015, Morrison et al. 2016), or it was stated directly that neonicotinoids are broad-spectrum (Moser and Obrycki 2009). Conversely, neonicotinoids were also distinguished as an "alternative" to or "substitute" for broad-spectrum

insecticides (Chuang and Hou 2008, page 352, 358) or as preferable to broad-spectrum insecticides (Ohnesorg et al. 2009). When we investigated whether these discrepancies might be related to different active ingredients within this subgroup, we found the very same issue, active ingredients being categorized both *as* and *as opposed to* broad-spectrum (Table 4.6).

Table 4.4 is the first time, to our knowledge, that a systematic inventory has been made of the insecticides that are being described as broad-spectrum or distinguished from broad spectrum. This is a useful contribution to support the clarification of the term, but the inventory itself does not necessarily lead to clarity on what belongs in the category or the rules for categorization. Linguistic uncertainty about the classification of neonicotinoids is highly consequential for the quality of communication among scientists and for the legitimacy of the use of our science in public and policy dialogue. Hence, it was concerning to see the unacknowledged contradictory categorization of neonicotinoid insecticides.

Definition via contrast and connotation

Broad-spectrum was used 255 times in the 60 recent papers we surveyed. We categorized its appearance into three types of use: a) as a stand-alone description, b) as a term in opposition or relation to another category, or c) as a term connoting positive or negative value to an insecticide. We found broad-spectrum was used as a stand-alone descriptor, unattached to any value-based connotations or in comparison to anything else 33.3% of the time (e.g., "Within these areas, use of broad-spectrum insecticides is higher in Fresno County than in Madera County" (Sisterson et al. 2008, p 1048)). In 40.4% of cases, broad-spectrum was used in relation or opposition to another category (e.g., "the impact of imidacloprid on predatory hemipterans was shown to be less than that of a broad-spectrum insecticide" (Ohnesorg et al. 2009, p 1823)). Broad-spectrum was used to connote the positive or negative value of an insecticide, through what we evaluated as desirable traits in 5.5% of uses and undesirable traits in 16.1% of uses. For example, "Foliar sprays of broad-spectrum insecticides applied during tree dormancy kill the majority of overwintering adults leaving few to infest new shoots in spring" (Khan et al. 2014, p 718) was considered desirable, and "broad-spectrum insecticides can negatively

impact the natural enemies associated with aphids" (Tran et al. 2016, p 2105) was considered undesirable. A small portion of uses (4.7%) encompassed more than one of our evaluative categories. For example, "Scientific literature indicates that most conventional broad spectrum insecticides are more harmful to beneficial insects than biorational spectrum compounds" (Rimoldi et al. 2012, p 1985) expresses both an undesirable trait, that broad-spectrum insecticides are harmful to beneficial insects, and also compares broad-spectrum insecticides to biorational spectrum compounds.

The term broad-spectrum was used most often in comparison to another category. This is problematic where authors imply the categories are mutually exclusive, yet that is not the case. Additionally, setting up these comparisons allows authors to imply membership and non-membership without actually saying such. For example, if a study states it compared imidacloprid to a broad-spectrum insecticide, the framing eases a reader into thinking that imidacloprid is *not* broad-spectrum (Table 4.6). When the boundaries of an insecticidal category, such as *broad-spectrum*, are ambiguous, vague, and/or relative, subjective decisions about how the category should be applied are required. Regan et al. (2008) categorize *subjective judgment* as an epistemic uncertainty, however, here we see the ambiguity, vagueness, and relative nature of *broad-spectrum* as contributing to *subjective judgment* in language. The high degree of flexibility for defining membership and non-membership of broad-spectrum and lack of clarification made it impossible in many cases to discern the mechanism(s) authors were using to categorize insecticides. Individuals designate which insecticides are and are not broad-spectrum and, as far as we found, there has been no discussion about dissimilar categorizations between papers. In this way, *subjective judgment* to determine broad-spectrum insecticide category membership is being used but not being acknowledged.

The second most common usage was to describe a noun unattached to any value-based connotations. While an author may think describing an insecticide as broad-spectrum is increasing the clarity of their work, using broad-spectrum as a descriptor decreases certainty if they do not define the term, since clearly there is no commonly understood and accepted meaning in our field. Additionally, we were unable to find a place listing which insecticides are and are not broad-spectrum (e.g., Table 4.4). The third

and fourth most common use of broad-spectrum was to connote undesirable and desirable qualities, respectively. This indicates that, at least in the last ten years, the category broad-spectrum generally carries more negative connotations. Thus, when framing research, it is possible to use the term intentionally or unintentionally to alter readers' perceptions of risk and benefit. In our inventory of terms that were used in association with broad spectrum (Fig. 4.2), many terms with negative valences (e.g., poison, toxicant) were associated with broad-spectrum and placed in opposition with alternatives described in positive terms (e.g., softer chemistries, organic, less active against humans, less toxic). To use *broad-spectrum* is thus to place something on a continuum of desirability.

Conclusion

As scientists, we strive to attain the greatest certainty in our work, yet uncertainty is inevitable. Linguistic uncertainty that arises when categorizing and describing insecticides illustrates the tension between reduced quality of information and more succinct communication. As an insecticidal category, *broad-spectrum* is compelling but uncertain. Despite this, or perhaps because of this, *broad-spectrum* is widely used in the entomological literature and can be a powerful descriptor. Despite only analyzing a small fraction of the papers that contain the words *broad-spectrum* and *insecticide*, we still found ample linguistic uncertainty. In all the papers we read, it was clear that authors did not feel the need to cite a primary source to define this term. We found that *broad-spectrum* as a category of insecticides is ambiguous, vague, relative, and subjective. Compounded, these linguistic uncertainties result in confusion about the bounds of this insecticidal category. This has led to specific insecticides and groups of insecticides being described both as and as not broad-spectrum, difficulty interpreting and comparing results across studies, and instability of the term's meaning over time. We hope this paper increases awareness of this linguistic uncertainty and recognition of the need to consider its effects on science and policy making. Additionally, we hope that, armed with the knowledge of where linguistic uncertainty is commonly found, and the effects of

linguistic uncertainty, entomologists can examine their own area of expertise for sources of linguistic uncertainty.

Addressing linguistic uncertainty broadly

There is no systematic way to identify or reduce all linguistic uncertainty. However, we can still seek to improve our communication across time and space. As a writer, you must know what you wish to convey, as well as how to communicate it with certainty. There is not a standard metric to measure or report the level of certainty your words embody. While it may not be possible to know all the other interpretations of your words, at the very least, it is important to know that there *are* other ways of interpreting even seemingly common terminology. Within a paper, the writer and the reader must come to a shared understanding of language and reality, often never having met each other. What assists this process is developing an appropriate shared context that the linguistic meaning of the paper is derived from. Carefully summarizing the pervious literature and defining problems and key terms can help establish or create a shared context within your paper. Though, as we have pointed out, many definitions using vague terms fall short of reducing uncertainty. Assessing the linguistic uncertainty in one's own work offers a lens of self-reflection onto the context that you are creating knowledge in. It would behoove the discipline of entomology to strike up a conversation about how we use, ignore, and feel about the linguistic uncertainty in our field.

Addressing linguistic uncertainty pertaining to insecticidal categorization

The categorizing mechanisms used in regulated and unregulated insecticide categories are not always understood, acknowledged, or considered deeply by writers who use these insecticide categories in their writing. We created Table 4.1 as a way to bring attention to this. We also would like to offer a framework to demarcate mechanisms for insecticidal categorization. Mechanisms are divided into three groups, process-oriented, outcome-oriented, and function-oriented, based on the insecticidal attribute(s) of interest. Process-oriented terms classify an insecticide based on how the insecticide is created or used. Outcome-oriented terms classify an insecticide based on the effects, from the population to biosphere scale, that the insecticide has when used. Function-oriented terms classify an insecticide based on its mode of action (i.e., effects at the individual

organism scale). For example, *organic* is a process-oriented categorization: whether an insecticide's ingredients are synthetically- or naturally-derived determines whether it is organic or not. Similarly, *biopesticide* is also a process-oriented categorization. In contrast, the US-EPA's *reduced-risk* pesticide category is outcome-oriented: the risks associated with the use of the insecticide, relative to current pest management options, is the criteria for membership. Risk includes potential to harm humans, birds, fish, honey bees, and beneficial insects (United States Environmental Protection Agency 2016). Similarly, the International Organization for Biological Control (IOBC) *level of harm* categories for insecticides (e.g., harmless, slightly harmful) are outcome-oriented categories as well. Function-oriented categories, such as the IRAC insecticide groups, sub-groups/chemical classes, and active ingredients, are classified based on the unique way by which the insecticide(s) effects organisms: pyrethroids are classified as sodium channel modulators because that is the mechanism by which they affect nerves and muscles (IRAC International MoA Working Group 2017).

When categories arise in scientific and public communication outside a regulating body, they often lack a uniform structure to determine membership or maintain oversight. Many of these non-formalized categories can be sorted into our three demarcation groups as well. *Synthetic*, *systemic*, and *integrated pest management-friendly*, are process oriented. *Environmentally persistent*, *broad-spectrum*, and *biorational* are outcome-oriented. *Toxic*, and *fast-acting* are function-oriented. Not all adjectives used to describe insecticides will fit perfectly into these three categories. For example, *cheaper*, *softer*, and *newer*. This system of demarcation is not intended to cover the entire range of potential descriptors, but rather to parse out the most common and widely used mechanisms of insecticidal categorization.

We created this framework because we saw in the literature a lack of understanding of the mechanisms used in delimitating certain boundaries of insecticide categories. Given that the determining factors of membership for most categories are unrelated, comparing insecticidal categories can set up false dichotomies. A false dichotomy occurs when two options are presented as mutually exclusive and the only possibilities, when in reality there are other possibilities and the categories may have

overlap. Our process-, outcome-, function-oriented framework highlights when categories created via different mechanisms are being compared. In such circumstances, when non-dichotomous categories are being compared, writers should attempt to determine what they are ultimately trying to test or say.

Addressing linguistic uncertainty pertaining to broad-spectrum

If broad-spectrum is used, and authors want to eliminate linguistic uncertainty associated with the term, they need to define the boundaries of broad-spectrum to the extent that allows a reader to independently determine the boundaries of the broad-spectrum category. Critical information to allow for this includes stating what *broad* is in relation to, specifying the context in which category membership was determined (e.g., crop system), noting which mechanisms (e.g., process-, outcome-, function-oriented) were used to define category boundaries, and offering an explicit definition of the term. The linguistic uncertainty of broad spectrum may at times be inconsequential, and reducing it unnecessary. Authors must determine in their papers individually how linguistic uncertainty effects the strength, validity, reproducibility, and long-term stability of their work.

Another aspect of uncertainty that must be address for broad-spectrum is its vague nature. We see two options regarding the vague nature of broad-spectrum. First, terms such as *wide*, *affect*, and *destroy*, that do not have clear boundaries, continue to be used in the explicit definitions of broad-spectrum insecticide. Broad-spectrum would remain a vague term and the boundaries of the category would remain difficult to determine. Consequences of this would likely follow the already occurring trends (e.g., contradictory insecticidal classifications, highly subjective nature of assigning membership).

Second, a concerted effort could be made to develop criteria that do not allow for cases along the border between being and not being a broad-spectrum insecticide. In this way, all insecticides could be sorted as either broad-spectrum, or not-broad spectrum. The process to do this involves first, deciding on one definition to work from. Let's say it was the Torre-Bueno definition "Relating to pesticides, killing a wide range of target organisms." Next, the vague words in the definition (i.e., killing, wide, target) would need to be defined with concrete delineating bounds. For instance, killing could be

defined as: complete mortality of at least 98% of the population in a treated area within one day of exposure, or functional mortality (i.e., inability to progress to the next life stage or produce offspring) of at least 98% of the population in a treated area within three weeks. The point is not what specific delineating bounds are arrived at, though across experts there likely would be strong opinions about what those bounds should be. The point is that there would need to be specific delineating bounds so that any person if given the set of criteria for membership and information about an insecticide, could determine if it was or was not broad-spectrum. There would likely also need to be strong institutional organization and power to make such a reform. Like Horowitz et al. (2009), we believe the EPA or a similar regulating body which can independently assess existing and future insecticides would need to be involved, and would need to make widely available the category membership criteria or a list of insecticides they determine to be members of the category. While it is entirely possible to do these things needed to make broad-spectrum not vague, the benefits and costs of doing so should be weighed by the community. How much good would it do to change only one of the many linguistically uncertainty insecticidal categories do? Is a complete overhaul of many of the terms needed? Will such a change actually take hold, or will change be resisted? What about all the papers which used the category before making it a clearly delimited category? How difficult would it be to find consensus among experts, or is consensus even needed? Which insecticidal attributes should be considered when creating the boundaries? Does the category need to be ecologically, economically, environmentally, and/or socially meaningful?

Ultimately insecticidal categories should be valid and relevant to allow for the most well-informed pest management decisions to be made. Conversations about utility and linguistic uncertainty across disciplines are necessary to clarify terminology. We would like to reiterate the need for an increased awareness of linguistic uncertainty in entomological writing broadly as well, and encourage mindful consideration of where and why linguistically uncertain words are being utilized

Tables and figures

Table 4.1. Categories of insecticides with a formalized structure, defined or certified by an organization.

Term(s)	Regulating body *	Characteristic(s) membership is based on?	Ambiguous	Vague	Defined in relative terms?
Reduced risk	USA-EPA	Risk in comparison to alternatives (United States Environmental Protection Agency 2016).	No	Yes	Yes, relative to other insecticides, dependent on the crop system.
Organic	OMRI	Physical components of insecticide ("OMRI Lists" 2016).	Not unless considering a global perspective	No	No
Insecticide group, subgroup/chemical class, and active ingredient	IRAC	Affected physiological system Mode of action classification informs the symptomology, speed of action and other properties of the actives therein (Insecticide Resistance Action Committee n.d.).	No	No	No
Level of harm (e.g., harmless, slightly harmful)	IOBC	Harm to natural enemies, where harm is evaluated as either mortality or a reduction in beneficial capacity (i.e., parasitism) compared to a control (Hassan et al. 1994)	No	Yes	Yes, relative to an untreated control.
Biopesticide	USA-EPA	Containing naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), or substances produced by plants containing added genetic material (plant-incorporated protectants) (United States Environmental Protection Agency 2017).	Yes	No	No

*Acronyms: US-EPA = United States - Environmental Protection Agency; OMRI = Organic Materials Review Institute; IRAC = Insecticide Resistance Action Committee; IOBC = International Organization for Biological Control

Table 4.2. Entomology journals that were included in our literature search for papers published from 2006-2016 that contained the terms *broad-spectrum* and *insecticide*. Journals were chosen based on their Thomson Reuters 2015 Journal Impact Factor and description, which needed to include some mention of agricultural entomology or pest management.

Journal	Impact Factor
Annual Review of Entomology	13.534
Journal of Pest Science	3.103
Current Opinion in Insect Science	2.719
Insect Science	2.551
Biological Control	2.012
Experimental and Applied Acarology	1.812
Agricultural and Forest Entomology	1.805
BioControl	1.767
Bulletin of Entomological Research	1.761
Journal of Economic Entomology	1.609
Journal of Applied Entomology	1.517

Table 4.3. Subset of definitions for the term *broad-spectrum.*

Term	Definition	Citation
Broad-spectrum	Relating to pesticides, killing a wide range of target organisms	A Glossary of Entomology (Torre-Bueno et al. 1989)
Broad-spectrum pesticide	"Chemicals that affect pests and beneficial organisms."	A Dictionary of Entomology (Gordh and Headrick 2011, page 221)
Broad-spectrum pesticide	"A pesticide that kills a large number of unrelated species."	University of California Agriculture and Natural Resources, Statewide IPM Program (University of California Agriculture and Natural Resources Statewide IPM Program 2016)
Broad-spectrum insecticide	"Broad-spectrum insecticides are effective against all insects, even the good ones."	National Pesticide Information Center (Oregon State University and US-EPA) (National Pesticide Information Center 2015)
Broad-spectrum insecticide	"Many insecticides are general purpose or wide range killers. These "broad spectrum" pesticides are used when several different kinds of insects are a problem. One chemical can kill them all. No broad-spectrum insecticide kills all insects; each varies as to the kinds of insects it controls."	Cornell University's Pesticide Safety Education Program (Cornell University Cooperative Extension 2012)
Broad-spectrum insecticide	"Insecticides affect various insects differently. An insecticide that is lethal against one group may have little, if any, effect on another. Generally, however, most insecticides kill many kinds of insects: broad-spectrum insecticides are those that kill more kinds than others."	The Pesticide Book (Ware and Whitacre 2004, page 82)

Notable references which lacked definitions of *broad-spectrum insecticide* include Encyclopedia of Entomology (Capinera 2008), foundational integrated pest management text: The integrated control concept (Stern et al. 1959).

Table 4.4. Insecticide resistance action committee (IRAC) insecticide classes and active ingredients and trade names stated to be or not be *broad-spectrum*. From this list membership to the *broad-spectrum insecticide* category is built.

IRAC level	Groups described as or synonymous to broad-spectrum insecticide		Terms found in both categories	Groups compared to or stated to be dissimilar from broad-spectrum insecticide	
Insecticide IRAC classes	carbamates diamides neonicotinoid	organochlorines organophosphate spinosyns	Diamides neonicotinoid the anthranilic diamide group	buprofezin diamides neonicotinoid	oxadiazine pyriproxyfen
Insecticide active ingredients or trade names	Acephate acetamiprid aldicarb azadirachtin (neem) azinphos methyl Besiege beta-cypermethrin Bifenthrin Carbaryl Chlorantraniliprole Chlorfenapyr chlorpyrifos cyfluthrin cypermethrin Dinotefuran endosulfan endosulfan	esfenvalerate Gusathion Imidacloprid lambda-cyhalothrin Maldison methomyl methyl bromide naled phorate Phosmet pyrethroid pyrethrum Spinetoram Temik Thimet α-cypermethrin	Acetamiprid Chlorantraniliprole imidacloprid Spinetoram	acetamiprid chlorantraniliprole emamectin benzoate flubendiamide imidacloprid indoxacarb methoxyfenozide pymetrozine spinetoram spinosad sulfoxaflor thiacloprid thiamethoxam	

Table 4.5. Examples of discrepancies in the literature between the insecticidal *activity* and insecticidal *category* for a given active ingredient. In most cases discrepancies occur across papers.

Active ingredient	Insecticidal activity	Insecticidal category
Spinetoram	"like other spinosyns, it **has broad-spectrum activity** causing hyperexcitation of the nervous system by activating the Da6 subunit of the nicotinic acetylcholine receptor while maintaining a safe environmental and toxicological profile" (Hamby et al. 2015, page 691)	**Reduced-risk** (Sial and Brunner 2010, Roubos et al. 2014, Hamby et al. 2015)
Chlorantraniliprole	"**provides potent and broad-spectrum activity** within the insect order Lepidoptera" (Wang et al. 2010, page 843)	**Anthranilic diamide group** (Wang et al. 2010) **Selective** (Whalen et al. 2016) **Reduced risk** (Sial and Brunner 2010, Hamby et al. 2015)
Acetamiprid	"**exhibited broad-spectrum insecticidal activity** that included toxicity to predatory beetles, predaceous and omnivorous bugs, green lacewings, predatory flies, and several moth species" (Rill et al. 2008, page 580)	**Broad-spectrum** (Rill et al. 2008) **Reduced-risk** (Roubos et al. 2014) **Not conventional broad-spectrum** (Wise et al. 2010)

Table 4.6. Categorization of active ingredients (AI) in the neonicotinoid insecticide class.

Insecticide category	AI(s)	Excerpt
Broad-spectrum	Acetamiprid	"The broad-spectrum neonicotinoid acetamiprid…" (Rill et al. 2008)
	Dinotefuran	"Dinotefuran is the third neonicotinoid which possesses a broad spectrum and systemic insecticidal activity." (Mu et al. 2016)
	Imidacloprid	"Sprays of Azadirachtin (Neem), Tropane (Datura), Spirotetramat, Spinetoram, and broad-spectrum Imidacloprid were evaluated to control ACP in spring and summer on 10-year-old "Kinow" *Citrus reticulata* Blanco trees producing new growth." (Khan et al. 2014)
Compared against broad-spectrum	Imidacloprid Thiamethoxam	"We compared three insecticides (imidacloprid, thiamethoxam, and pymetrozine,) to a broad-spectrum insecticide (lambda-cyhalothrin) and an untreated control using two application methods" (Ohnesorg et al. 2009)
	Acetamiprid	"The reduced-risk products, acetamiprid and spinetoram, were as toxic as some of the broad-spectrum insecticides."(Roubos et al. 2014)
	Acetamiprid Imidacloprid	"For *H. convergens*, indoxacarb, acetamiprid, imidacloprid, and the broad-spectrum insecticides caused significant acute effects" (Roubos et al. 2014)
Distinguished from conventional broad-spectrum	Thiacloprid Acetamiprid	"The neonicotinoids thiacloprid and acetamiprid demonstrated strong larvicidal and ovicidal activity but were somewhat weaker adulticides than the conventional broad-spectrum compounds" (Wise et al. 2010)

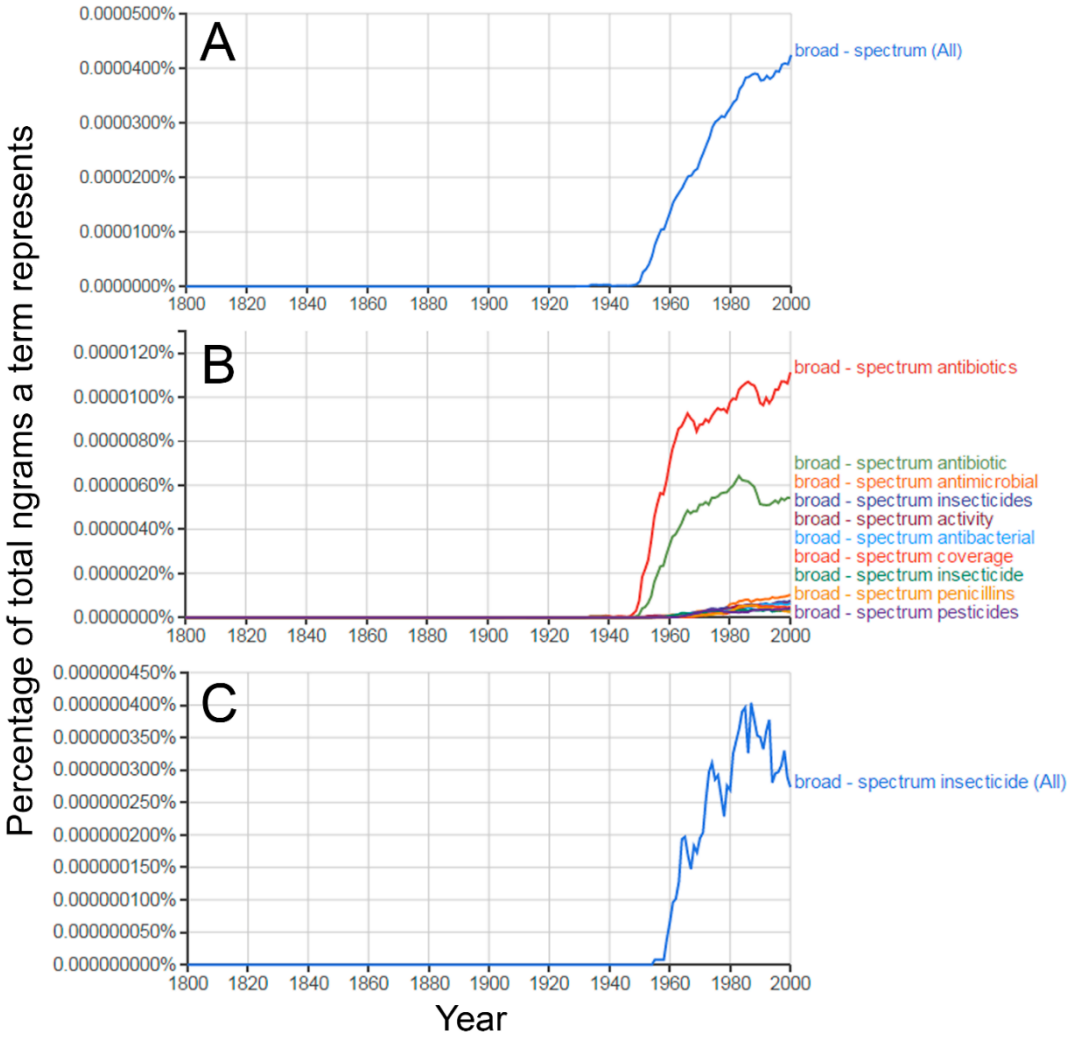

Figure 4.1. Relative frequency of use of **A)** broad-spectrum, **B)** top ten words that follow the term broad-spectrum, and **C)** broad-spectrum insecticide in books scanned by Google through time.

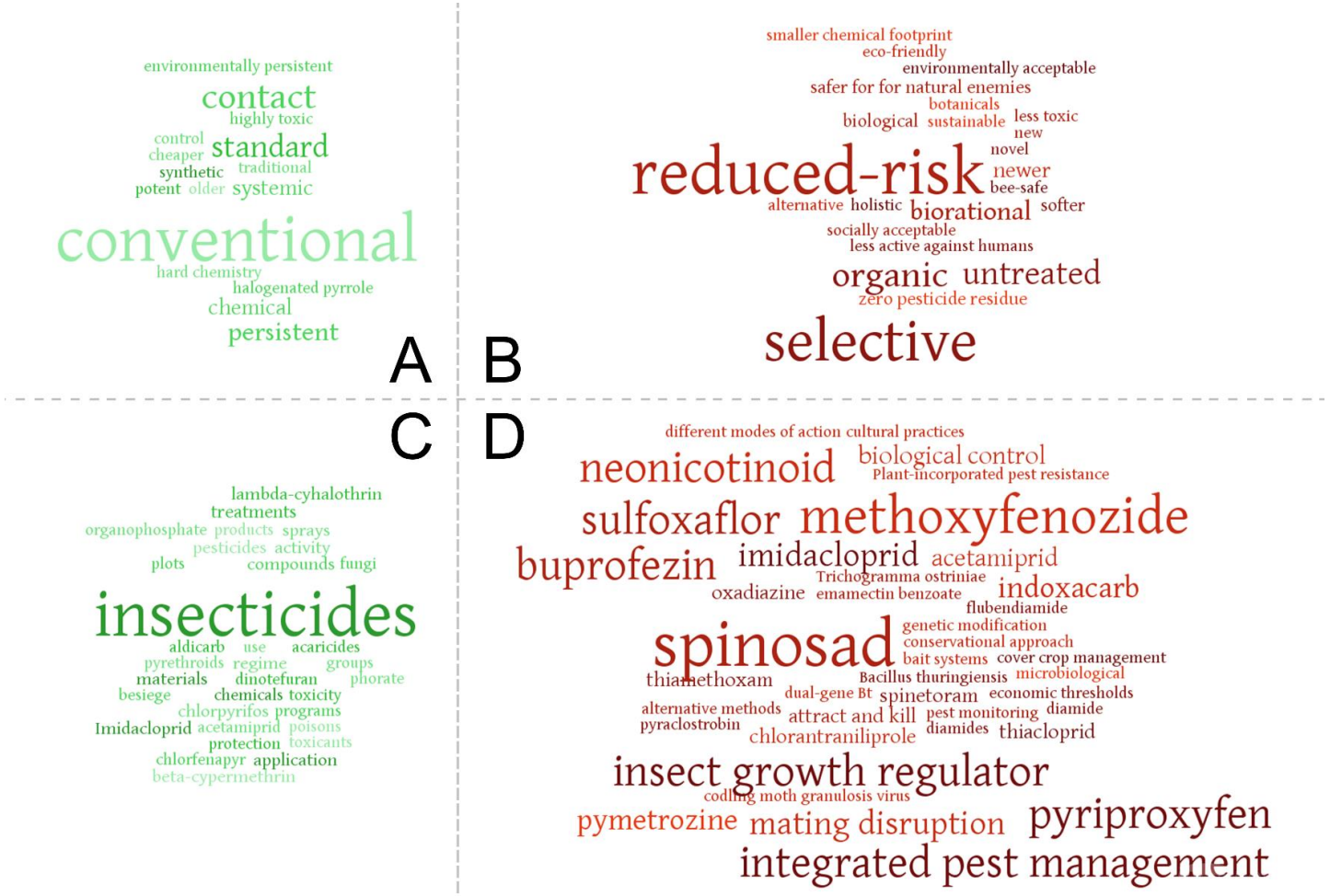

Figure 4.2. Adjectives **A)** associated and **B)** disassociated from *broad-spectrum* and nouns **C)** associated and **D)** disassociated from *broad-spectrum*. Size of the word represents relative frequency of its use.

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Appendix 1: *Halyomorpha halys 4th instar* mortality and sublethal molting effects following insecticidal exposure

Here we present a preliminary laboratory insecticide bioassay testing the effects of four insecticides on *H. halys* 4th instars. Mortality and molting to 5th instar were measured after residual and topical insecticidal application.

Materials and methods

Halyomorpha halys 4th instars were exposed to insecticides either residually or topically. For residual exposure, four nymphs were placed together in a Petri dish (100 x 15mm) that had been previously sprayed and allowed to dry completely (approximately 1 h dry time). After 24 h of residual exposure (with no food or water), the nymphs were removed and placed together in clean, lidded tubs (Translucent 473 ml, Consolidated Plastics Stow, OH), provisioned with dry organic soybean seeds (*Glycine max* (L.) Merr. *ad libitum* and a cotton ball soaked in water. Cotton balls were re-wetted as needed. Each residual exposure treatment was replicated three times, totaling 12 nymphs/treatment. For topical exposure, four nymphs were placed together in an unsprayed Petri dish. Coarse wire mesh (0.64 cm × 0.64 cm) was placed over the Petri dish, and the dish, nymphs, and mesh were sprayed. After 1 h in the treated dishes (with no food or water), nymphs were removed and placed together in a clean tub as described above. Each topical exposure treatment was replicated four times, totaling 16 nymphs/treatment. Individuals were assessed 1 hour after exposure and then daily for 7 days.

Four 4th instars in a Petri dish were considered the independent experimental unit. Generalized linear effects models with a binomial error distributions were used to test the effects of replication and insecticide treatment on: 1) mortality at 7 DAT, and 2) the proportion of 5th instars at 7 DAT. There were not enough degrees of freedom to test the interaction of replication and insecticide treatment. Proportion of 5th instars was calculated by dividing the number of 5th instars by the number of living nymphs (both 4th and 5th instars) at 7 DAT. Low replication levels (i.e., less than four) for topical exposure prevented use of generalized linear mixed models. Data were analyzed separately for

residually- and topically-exposed 4th instars. Tukey's HSD (multcomp, *cld*, *glht* (Hothorn et al. 2008)) was used to determine significant differences ($\alpha = 0.05$) between treatments.

Results

Direct mortality

Residual exposure to insecticide affected mortality of 4th instars at 7 DAT ($\chi^2 = 41.89$, $df = 4$, $P < 0.0001$), but replication did not ($\chi^2 = 0.14$, $df = 2$, $P = 0.93$). For topically-exposed 4th instars, insecticide treatment affected mortality of 4th instars at 7 DAT ($\chi^2 = 46.73$, $df = 4$, $P < 0.0001$), but replication did not ($\chi^2 = 4.64$, $df = 3$, $P = 0.20$). Mortality increased numerically over time for all insecticides and routes of exposure except when 4th instars were treated with azadirachtin + pyrethrins (Fig. 1). Both residual and topical exposure to azadirachtin + pyrethrins resulted in an initial knockdown of individuals; at 1 h and 1 DAT some individuals treated with azadirachtin + pyrethrins were moribund or appeared dead, but subsequent measurements at 2 DAT and beyond showed the individuals had recovered (Fig. 1). At 7 DAT, residual exposure to sulfoxaflor and bifenthrin resulted in significantly higher mortality 0.92 ± 0.08 (SEM), and 1.00 ± 0.00, respectively, than the untreated controls at 0.00 ± 0.00 (Fig. 1a). Topical exposure to azadirachtin + pyrethrins, sulfoxaflor, and bifenthrin resulted in significantly higher mortality 0.94 ± 0.06, 1.00 ± 0.00, and 1.00 ± 0.00, respectively, than the untreated controls (0.19 ± 0.10) (Fig. 1b). In general, topical exposure of insecticides caused mortality in treated 4th instars sooner, and in greater proportion, than residual exposure (Fig. 1).

Molting

Residual exposure to insecticide affected the proportion of surviving nymphs which molted to 5th instar by 7 DAT ($\chi^2 = 9.42$, $df = 2$, $P = 0.009$), but replication did not ($\chi^2 = 2.62$, $df = 2$, $P = 0.27$). Neither topical exposure to insecticides ($\chi^2 = 0.12$, $df = 1$, $P = 0.73$) nor replication ($\chi^2 = 2.50$, $df = 4$, $P = 0.48$) affected the proportion of 5th instars at 7 DAT. Residual exposure to azadirachtin + pyrethrins significantly reduced the proportion of 5th instars at 7 DAT compared to the control (Table A1).

Tables and figures

Table A1. Observed proportion of surviving *H. halys* nymphs that molted to 5th instar by 7 days following residual or topical exposure of 4th instars to insecticides.

Insecticide	Proportion ± SEM	
	Residual exposure (n)	Topical exposure (n) *
Control	0.92 ± 0.08 (12) a	0.86 ± 0.09 (14)
Azadirachtin + Pyrethrins	0.29 ± 0.17 (7) b	1.00 ± 0.00 (1)
Spinosad (Organic-certified)	0.82 ± 0.12 (11) ab	0.90 ± 0.09 (10)
Sulfoxaflor	1.00 ± 0.00 (1) NA	0.00 ± 0.00 (0)
Bifenthrin	0.00 ± 0.00 (0) NA	0.00 ± 0.00 (0)

* Insecticide treatment was not a significant main effect in the topical-exposure model so no post-hoc differences of means were calculated.

Different letters within a column indicate significant differences of means ($\alpha = 0.05$).

Each proportion represents the mean across three replications for residual exposure and four replications for topical exposure.

Numbers in parentheses indicate sample size (number of surviving individuals). Means noted as NA were not included in means comparisons due to complete mortality or low sample size.

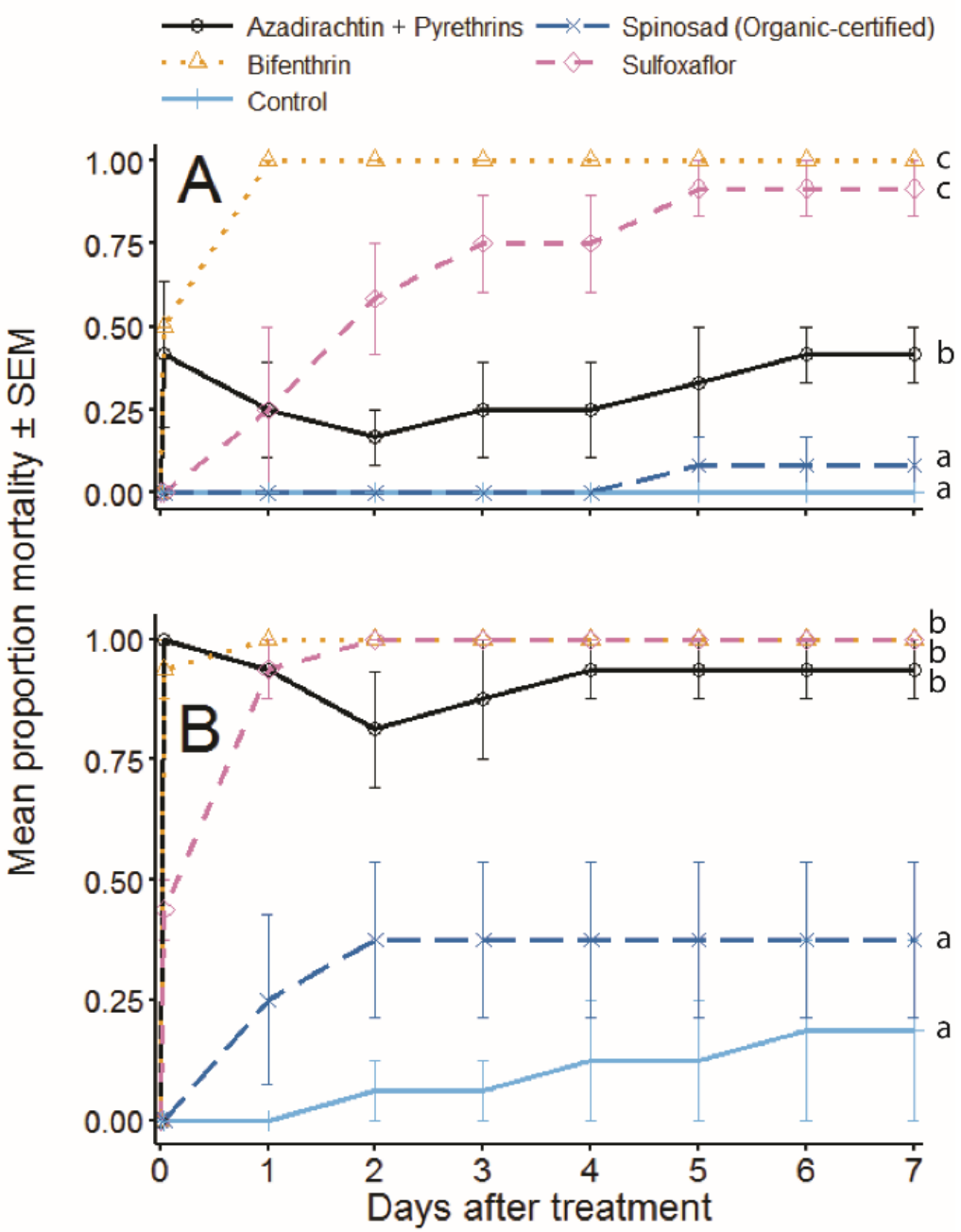

Figure A1. Proportion mortality of 4th instar *H. halys* after being exposed **A)** residually for 24 h or **B)** topically to insecticides. Each symbol represents the observed mean across a) three replications, and b) four replications. Different letters within a graph indicate significant differences of means at 7 d after treatment ($\alpha = 0.05$). Mortality was determined by an inability to walk.

Appendix 2: Effect of starvation on supercooling points

Food in the gut can create ice nucleation sites, potentially causing insects to have higher supercooling points than if their gut is empty of food (Salt 1958b). We tested whether two days of starvation had an effect on the supercooling points of non-diapausing adult *H. halys.*

Materials and methods

To imitate a scenario of *H. halys* voiding its gut, we starved adults (reared at 25°C 16L:8D) for two days, though still provided water. After starvation, we measured supercooling points using contact thermocouple thermometry. Individual adults were placed in close proximity to coiled copper-constantan thermocouples (e.g., Hanson and Venette 2013) that were attached to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Temperatures were recorded once per second and logged by using Tracer-DAQ software (Measurement Computing, Norton, MA). We identified the lowest temperature reached before the exotherm, or spontaneous release of heat indicative of a phase change from liquid to solid, to denote an individual's supercooling point. The insect and thermocouple were confined in a 20 or 35ml syringe (Monoject syringes with leur lock tip) that was placed at the center of a 20 x 20 x 20cm polystyrene cube and then into a -80°C freezer where the insects cooled at a realized rate of -0.82ºC (± 0.008°C) per minute according to Carrillo et al. (2004).

Linear regression was used to test the effect of sex, starvation, and the interaction of the two on *H. halys* supercooling points and mass. Backward elimination (i.e., stepwise removal of non-significant model terms from most to least complex) was used to determine model parameters. Each iteration of a model was tested for assumptions of normality and heteroscedasticity via a Shapiro-Wilk normality test and Breusch-Pagan test (car, *ncvTest* (Fox and Weisberg 2011b)) respectively. Supercooling points and masses were transformed with a Box-Cox power transformation (supercooling point $\lambda = -0.7878$, mass $\lambda = -0.0606$) to meet model assumptions ($p > 0.01$) [MASS, *boxcox* (Ripley

and Venables 2002)]. Welch two sample t-tests were used to compare means between sexes and between starvation treatments.

Results

No significant differences in mean supercooling points were found for main effects of sex, starvation, or the interaction of the two (Table A2). The effect of the interaction of sex and starvation on mass was not significant. Starvation did significantly affect mass (starved: 0.1069 ± 0.0081 g (n=28); un-starved 0.1314 ± 0.0074 g (n=38)). This confirms that, within the starvation period we chose, insects did expel food from their gut. Sex also affected mean mass (females 0.1466 ± 0.0081 g (n=34); males 0.0938 ± 0.0041 g (n=32)), males are commonly known to be lighter than females.

Tables

Table A2. Supercooling points of adult *H. halys* did not differ based on sex, starvation, or the interaction of the two.

Days starved	Sex	Mean SCP ± SEM °C (n)
0	F	-13.57 ± 0.78 (19)
	M	-12.63 ± 0.56 (19)
2	F	-13.50 ± 0.74 (15)
	M	-14.77 ± 0.62 (13)